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PHYSICO-CHEMICAL STUDIES ON THE ACTIVATION AND FERTILIZATION OF FISH EGGS

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INTRODUCTION

Generally speaking, the studies on the development of fish eggs have been carried out from three main standpoints, viz., morphological, physiological and chemical ones. Among these studies, the morphological analysis of fertilization seems now to be fairly complete. In this field of study, besides the works of several earlier investigators, Ozima's ('43) study on carp egg and K. Yamamoto's ('52) on dog salmon have recently been reported. It is obvious that physiological studies furnish an important means for the analysis of the fertilization phenomena. Students making this approach have mainly focused attention on the cortical change at the time of fertilization. According to T. Yamamoto ('44a, 44b), numerous cortical alveoli embedded in the cortical layer of unfertilized eggs of medaka (*Oryzias latipes*) break down at fertilization and bring about activation in a wave-like manner. Similar phenomena have been confirmed in the eggs of other fishes, such as salmons (*O. narka* and *O. masou*), the trout (*S. irideus*) and roach (K. Yamamoto '49, '50, '51 and Kanoh '50) and carp, smelt and goldfish, etc. (T. Yamamoto '51, '54). The cortical change is also induced by mechanical, electrical and chemical stimulation (T. Yamamoto, '39, '47). T. Yamamoto ('39, '44a, '44b, '51) has advanced the theory of the "fertilization wave" on the basis of his own numerous physiological results concerning the cortical change. K. Yamamoto ('50) and Kusa ('50) independently of each other have reported that the unfertilized dog salmon egg is readily activated merely by being brought in contact with water and that the cortical change is induced.

They then confirmed that there exists an important correlation between breakdown of cortical alveoli and fertilizability. Although many works have been published on the cortical change at the time of activation, this branch of study seems still to leave many questions suitable for further research, because the fertilization process is highly complicated and the progress of the process is dependent upon a number of factors which include biological and biochemical characters.

The gaps which remain untouched in morphological and physiological studies on fertilization should be filled up by chemical studies. In former times, biochemists considered the egg only as a convenient material for the investigation of certain chemical phenomena under comparatively simple condition, and naturally they have paid no attention to the physiological changes of the egg. But, now, in consideration of the morphological and physiological results, the writer has undertaken to make clear from the viewpoint of chemistry the fertilization reaction of fish egg. For this branch of study, it seems prerequisite to clarify the chemical nature of fish egg, but that problem is not yet completely solved, as may be learned by consultation of Needham's books ('31, '42). Recently, Kusa ('53, '54) demonstrated the presence of a polysaccharide substance within the cortical alveoli of the stickleback and the salmon eggs by cytochemical techniques. Furthermore, the existence of a lipid wall which surrounds

the cortical alveolus has been reported by Aketa ('54) in the egg of medaka (*Oryzias latipes*). In the same material, T. Yamamoto ('51) found minute cortical granules embedded in the surrounding cytoplasm of the cortical alveoli. He suggests that an esterase-like substance may be contained in the granules which participates in the activation process. These findings about the chemical nature of the alveoli may present an important clue as to the mechanism of fertilization reaction.

The present paper deals with the physico-chemical nature of the egg at the time of activation or of fertilization. Especially, the writer has investigated the changes in protein and lipid of eggs at the time of fertilization and the interaction between the sperm and protein components of the egg.

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MATERIALS AND METHODS

In this experiment, the dog salmon (*Oncorhynchus keta*), rainbow trout (*Salmo irideus*), pond smelt (*Hypomesus olidus*) and crucian carp (*Carassius auratus*) were used as materials. The dog salmon were taken at the Chitose, the Yagumo and the Shiriuchi hatcheries in Hokkaido. The pond smelt and crucian carp were collected from the Ōnuma in Hokkaido. The rainbow trout were collected at "Nanae fish-culture pond" which is located at Nanae, a suburb of Hakodate.

The isotonic Ringer used here has the following constitution; M/7.5 NaCl 100

parts + M/7.5 KCl 2 parts + M/11 $CaCl_2$ 2.1 parts, pH is regulated at 7.0 by adding of N/10 $NaHCO_3$ solution. This isotonic Ringer solution was prepared with reference to the paper of Aoki ('39). The eggs taken from a single female were used for a series of experimemts except in the case of the eggs of pond smelt.

When the eggs of dog salmon and rainbow trout were reserved in refrigerator or in thermostat at 0°～5°C, the fertilizability of the eggs was retained unaltered at least for three days. In case of sperm, its vitality was retained for about 12～18 hours. The vitality of the pond smelt and crucian carp eggs was less than that of the dog salmon and rainbow trout. Both biological and chemical analysis were made in parallel. For instance, response of the cortical alveoli to the various enzyme-activators and -inhibitors has been microscopically observed, together with chemical analysis of enzyme activity in the eggs. Protease activity was measured mainly by KOH-alcohol microtitration and Anson's method ('38). Esterase activity was measured by the falling drop method using a stalagmometer. Electrophoretic studies on the protein were undertaken with HT-B type Tiselius apparatus. The details of experimental methods are described in each section.

EXPERIMENTAL RESULTS

I Chemical nature of the inner swelling layer of egg membrane and conjugation between eggs and spermatozoa in the dog salmon

1) *Chemical nature of the inner swelling layer of egg membrane*

The egg membrane of the dog salmon consists of three layers, an outer hyaline layer, middle layer and inner layer. The hyaline layer was found by Aoki who named it "Tomeiso." The membrane which has never been in contact with water, readily swells up in acid solution, but such swelling does not occur in a membrane having already absorbed water. The inner layer of the chorion, however, swells up more vigorously than the outer and finally disperses in acid solution (Aoki '41).

In the present study, the protein nature of the inner swelling layer of the chorion has been investigated. The inner swelling layer solution was prepared as follows; one hundred ripe unfertilized eggs were cut with small scissors and then washed repeatedly with isotonic NaCl solution to remove yolky substances. The washed egg membranes were immersed in 100 ml of 0.01 N HCl solution and then filtrated to remove undissolved residues. The mucus solution thus prepared contains mainly inner layer substance of the membrane. The qualitative protein test and glycoprotein reaction tests were made on the mucus solution.

As shown in Table 1, protein reactions, especially the precipitation reactions with acids, concentrated neutral salt solutions and heavy metals, were clearly positive. This sample solution was also precipitated by the addition of lead-acetate or calcium chloride. From the above facts, together with the nature of its swelling in acid

Table 1. Qualitative protein test of swelling substance of egg membrane in the dog salmon egg.

Method		Reaction*
Color reaction	Biuret reaction Xanthoprotein reaction Hopkins & Cole's reaction Leiblmann's reaction	– – – –
Coagulation reaction by heat		–
Coagulation reaction by acid	Hydrochloric acid Sulphuric acid Nitric acid	+ + +
Precipitation reaction by concentrated neutral salt solution	Ammonium sulphite Potassium chloride	+ +
Precipitation reaction by solvents	Alcohol Acetone	– –
Precipitation reaction by heavy metals	Cupric sulphate Silver nitrate Lead acetate	+ + +

* + : Positive reaction, – : Negative reaction.

solution, the writer was made aware that this inner swelling layer might be composed of glycoprotein. Thus, following experiments were made to ascertain the reasonableness of the supposition.

i) After hydrolysis with 2% HCl, the inner swelling layer solution readily gave a positive Fehling reaction. But this reaction was negative in the solution which was not hydrated.

ii) The presence of sulphur in inner layer solution was confirmed qualitatively by use of barium chloride.

iii) The organic nitrogen was detected by micro-Kjeldahl's method.

iv) From these facts, it may be concluded that the inner layer of egg membrane is mainly composed of glycoprotein. Further, the evidence that the mucus solution was not precipitated with dilute acetic acid, suggests that it must be a kind of mucoid. Further, tyrosin which is, in general, richly contained in the egg membranes of fishes, and tryptophane could not be detected qualitatively.

2) *The conjugation between eggs and spermatozoa*

As stated above, the inner layer of the egg membrane is composed mainly of glycoprotein (acid protein). On the other hand, salmine, so-called alkaline protein, is a main chemical constituent of the sperm. In this section, the writer deals with the roles and characters of the outer and inner layers of the chorion and of the egg fluid at the time of the attachment of the spermatozoa to the eggs.

a) The effects of salts, hyaline layer substance and egg fluid on the conjugation between eggs and spermatozoa

When the egg fluid and some amount of the outer layer substances were removed from the eggs with the aid of filter paper, the fertilization rate of the eggs decreased conspicuously, but these eggs could be caused to recover their fertilizability by the addition of a small volumes of isotonic Ringer or egg fluid to the eggs (Table 2). These findings show that the egg fluid plays a part at the instant of conjugation between eggs and spermatozoa.

Table 2. Effects of outer layer substance and egg fluid on the fertilization of the dog salmon egg.

Method of treatments of the egg	Fertilization rate (%)	Agglutination of sperm*
Control	99	−
Inseminated after removed the outer layer substance and egg fluid	33	+
Added again small amount of egg fluid after removed the outer layer substance and egg fluid, and inseminated	91	−
In above case, added small amount of isotonic Ringer's solution instead of egg fluid, and inseminated	92	−

* + : Positive agglutination of sperm, − : Negative agglutination of sperm.

Owing to the difference of protein constituents, semen, egg fluid, outer hyaline and inner layers of the egg membranes give a different response to the acid or alkaline solutions respectively. In detail, semen, egg fluid and hyaline layer solution were not precipitated by the addition of N/10 $NaHCO_3$ solution, but they were precipitated by N/100 HCl solution. On the contrary, the inner swelling layer solution (regulated at pH 7.0) was not precipitated by acid.

The agglutination of spermatozoa is considered as an essential factor at the time of conjugation between spermatozoa and eggs. The semen was agglutinated with neither isotonic NaCl nor KCl, but it was agglutinated by the addition of isotonic $MgCl_2$, $CaCl_2$ and $AlCl_3$. The agglutinative character of semen becomes stronger along with the increase of ionic value of salts. The agglutinative reaction of semen to isotonic $AlCl_3$ was strongest. The coagulative reaction of the hyaline layer substance to salts solutions was similar to that of semen. The egg fluid was not coagulated by the above salt solutions, even by 1 M $CaCl_2$. The inner swelling layer solution was coagulated by more concentrated solution than M/11 $CaCl_2$ which is nearly isotonic with the egg content.

The semen of the dog salmon is mainly composed of salmine (protamine). Salmine is also coagulated by the addition of isotonic $CaCl_2$ and $MgCl_2$ just as semen is, but arginin which constitutes the main component of the salmine was not coagulated by the above mentioned salt solutions.

As stated above, the coagulative reactions of various parts of the egg membrane

and of semen to salt solutions differ respectively.

b) The role of calcium ions

Among various salt ions, Ca ions are considered to be necessary for the fertilization processes of fish egg, as has already been clarified by an excellent work of T. Yamamoto ('54). The present paper deals with the role, origin and character of Ca in the fertilization process of the dog salmon eggs. The eggs treated with Ca-free Ringer's solution for 20 minutes become unfertilizable, but if they were put back into the Ringer's solution, their fertilization took place quite normally (Table 3).

Table 3. Changes in fertilization rate by treatment with Ca-free Ringer, Na_3-citrate, Na-acetate and by removal of egg fluid of the superficial part of egg.

Method of treatments of the egg	Fertilization rate (%)
Control	98
Treated with Ca-free Ringer	17
Transferred to Ringer after immersed in Ca-free Ringer	91
Added distilled water after insemination by dry method	93
Inseminated by dry method after removal of egg fluid, and added distilled water	57
As above case, added tap water instead of distilled water	59
Inseminated by dry method after treated with isotonic Na_3-citrate (M/15) for 10 min., and added distilled water	21
As above case, added tap water instead of distilled water	21
Transferred to isotonic $CaCl_2$ after immersed in Na_3-citrate (M/15), and added distilled water after insemination by dry method	79
As above case, added tap water instead of distilled water	90
Treatment with isotonic Na-acetate	97
Transferred to isotonic $CaCl_2$ after immersed in isotonic Na_3-citrate	93

This fact shows that Ca located in the superficial parts of eggs diffuses into the Ca-free Ringer's solution, but the eggs can readily take back the lost Ca from the Ringer's solution. The same is true in the solution of Na_3-citrate whose anions serve to precipitate of Ca. That is to say, the eggs lose their fertilizability by treatment with isotonic Na_3-citrate (M/15), but those retreated with isotonic $CaCl_2$ (M/11) showed an almost normal percentage of fertilization (Table 3). This fact also indicates that the Ca of eggs combines with the anions of Na_3-citrate solution and stable Ca-citrate is formed, and further that by the transfusion into the isotonic $CaCl_2$ solution the eggs can obtain the Ca necessary for fertilization.

These ideas will be supported by the following evidence that the Ca-volume in media is clearly increased by immersion of eggs into Ca-free Ringer or isotonic Na_3-citrate (Tabel. 4). The Ca-content of the medium was measured by the usual weight method. The result indicates the dispersion of Ca into the medium from the eggs. Furthermore, the following experiment may give evidence supporting the above idea.

Table 4. Changes in Ca-content of media before and after immersion of eggs.

Solution immersed	Ca-content of medium (A) after immresion (mg)	Ca-content of medium (B) before immerstion (mg)	(A)-(B) (mg)
in Ca-free Ringer	7.1	5.6	1.5
in isotonic Na_3-citrate	33.6	28.5	5.1

1000 eggs were immersed in 700 ml of Ca-free Ringer or isotonic Na_3-citrate solution for one minute, and were removed by filtration. Ca-content of media was measured by weight method using ammonium oxalate as precipitants.

Even after treatment for 10 minutes with isotonic Na-acetate whose anions are ionized perfectly, the eggs were fertilized normally (Table 3). This seems to show that although the formation of Ca-acetate due to the combination of the Ca of eggs with the anions of solution is expected, the substance is unstable and the ionization of Ca-acetate takes place at the time of insemination. As to the source of Ca ions necessary for fertilization, there is a question as to whether Ca ions contained in semen participate in fertilization or not. When dry semen is mixed with egg fluid, the fertilizability of sperm is preserved for a long time (Ellis & Jones '39). In this experiment, egg yolk prepared from the unfertilized eggs was used instead of the egg fluid. Since the dry insemination by means of fresh semen mixed with egg yolk which was washed twice during 10 minutes with Ca-free Ringer's solution did not influence the fertilization (Table 5), it may be said that the Ca of semen does not play any part in the fertilization process. Further the fertilization took place normally in distilled water, and also only about 50 per cent of eggs were fertilized by the removal of the

Table 5. Changes in fertilization rate by removal of Ca present in semen.

Method of sperm treatment	Fertilization rate (%)
Mixed with egg yolk (blank test)	67
Mixed with egg yolk and washed with isotonic Ringer	75
Mixed with egg yolk and washed with Ca-free Ringer	77

Table 6. Changes in Ca-content of swelling layer of the egg membrane after immersing in distilled water and in tap water.

Solution used for egg	Dry weight of swelling layer substance (g)	Ca-content (mg)	Ca-content / Dry weight (%)
Swelling layer of the unfertilized egg (control)	0.568	1.6	0.29
Distilled water	0.015	1	6.6
Tap water	0.015	1	6.6

The swelling layer substsnce was dissolved in N/100 HCl, evaporated and dried. This dried swelling substance was dissolved in hydrochloride and precipitated with 5 % ammonium oxalate. The precipitated calcium oxalate was dissolved again in 0.01 N NaOH and titrated with 0.01 N HCl. The Ca-content was calculated from this titration value.

surface fluid (body fluid) of eggs with aid of filter paper (Table 3).

Therefore, it is most probable that Ca ions used in the conjugation between the sperm and eggs are originated from the superficial part of the egg. On the other hand, some physical change in the egg membrane has occurred at the time of activation. The Ca-content of egg membrane was increased by activation of the egg. That is to say, the inner layer of the egg membrane which fully absorbs water (activated egg membrane) is richer in Ca-content than that having not been in contact with water (unfertilized egg membrane) (Table 6). Ca ions may participate in the changes in egg membrane at the time of activation. The Ca-contents of the swelling layer were measured by the usual volumetric method.

II Lipid and oil at the time of activation of the eggs of dog salmon and crucian carp

1) *The response of the cortical alveoli to both esterase-activator and -inhibitor*

As stated above, the breakdown of cortical alveoli is brought about by the activation of the egg. T. Yamamoto ('44a, '44b, '47, '49) who worked on this subject in detail, using *Oryzias* eggs as the material, has found minute cortical granules embedded in the surrounding cytoplasm of the cortical alveoli, and suggested that an esterase-like substance in the granules may participate in the activating reaction. Cortical alveoli are also observed in the unfertilized egg of the crucian carp.

In the present study some experiments on the response of the activating and inhibiting enzyme at the time of the activation of eggs has been carried out. The existence of the lipase (esterase) in the egg is also to be confirmed.

a) The response of the cortical alveoli to the esterase activator

The ripe egg of the crucian carp loses its fertilizability within 40 seconds in fresh water at 13°∼15°C. The cortical alveoli of the unfertilized egg disappear in 13∼15 seconds after immersion in water and perivitellin space appears in succession. Among various esterase activators, lead-acetate gave good results in the experiments. When the unfertilized ripe egg was treated with 0.01% Ringer-lead-acetate solution and returned to isotonic Ringer's solution, the breakdown of the cortical alveoli and subsequent elevation of the chorion ensued. The breakdown of the cortical alveoli was also caused by 0.005% Ringer-pancreatin solution containing esterase. However, if the unfertilized ripe egg is immersed in this solution for 15∼18 minutes, a part of the chorion is dissolved out.

b) The response of the cortical alveoli to the esterase inhibitor

Physiologically, the response of the cortical alveoli to the esterase inhibitor is of more interest than their response to an activator. The breakdown of the cortical alveoli of the unfertilized ripe egg occurred just at the moment of treatment with

distilled water. Such a method as treatment with water was most effective for the activation of the eggs. However, when the unfertilized ripe egg was immersed in 0.001% Ringer-monoiodo-acetate solution for 5 minutes, then washed with isotonic Ringer's solution and transferred to distilled water, the breakdown of the cortical alveoli did not occur until 60~90 seconds had elapsed.

The effect of 0.01% Ringer-lead-phosphate solution was the same as that of the above solution. Furthermore, in the egg treated with esterase inhibitor and transferred to the esterase activator, the breakdown of the cortical alveoli took place more slowly than in the egg immersed in water directly. The unfertilized ripe egg was first immersed in 0.01% Ringer-monoiodo-acetate, then washed with isotonic Ringer's solution and finally immersed in 0.01% Ringer-lead-acetate. In this experiment the breakdown of the cortical alveoli occurred after 5~6 minutes and the elevation of chorion ensued. The experiment using lead-phosphate instead of monoiodo-acetate also yielded the same result.

c) The response of the cortical alveoli to the decomposed substance of esterase

It seems probable that the decomposed substance of esterase exerts influence upon the activation process of the egg. As the decomposed substances of esterase (especially, lipase and lecithase), fatty acid, alcohol, cholin, lysolecithin, phosphoric acid, glycerophosphoric acid and glycerin are produced. Of these substances, fatty acid (stearic acid and oleic acid), alcohol, lysolecithin, phosphoric acid and glycerophosphoric acid have been employed. Lysolecithin was prepared from fresh hen's egg by means of Nikuni's method ('32).

Activation did not occur in such solutions as 0.1% Ringer-alcohol, 0.01% Ringer-phosphoric acid and 0.01% Ringer-glycerophosphoric acid. The solution of 0.003% Ringer-lysolecithin also had no effect. The lack of response of the cortical alveoli to the lysolecithin may be accounted for by the low concentration of the solution. Of fatty acids which affect the egg, oleic acid and stearic acid were used. When the unfertilized ripe egg was immersed in 0.01% Ringer-oleic acid solution for 2~3 minutes and then returned to isotonic Ringer, the cortical alveoli disappeared; subsequently the perivitellin space was formed. On the other hand, the egg showed no change even upon treatment with 1% oleic acid solution for 60~90 seconds. However, if the egg is returned to isotonic Ringer's solution after washing several times with the same solution, the breakdown of the cortical alveoli takes place subsequently. The same result was also obtained with stearic acid. But the effect of stearic acid on the egg is stronger than that of oleic acid, because the egg immersed in 1% stearic acid solution for more than 40 seconds, showed cytolysis.

d) The confirmation of lipase (esterase) activity in the egg

In previous parts, the effects of esterase on activation of the egg have been

examined. Nextly, it seems necessary to ascertain whether or not esterase is contained in the egg itself. The lipase (esterase) was obtained from the egg a modification of Rona and Michaelis' method ('11). The method of preparation was as follows. The enzymes prepared from both the unactivated (unfertilized) and the activated eggs showed the same activity due probably to the activation of the enzyme obtained from the unfertilized egg in the processes of enzyme preparation. Activated eggs immersed in distilled water for 10 minutes were ground in a mortar and mixed with four to five volumes of isotonic M/7.5 NaCl solution. This suspension was centrifuged after being kept standing for 12~16 hours at room temperature (10°~15°C). The supernate was fractionated by precipitation at 60 per cent saturation of ammonium sulphate and the fraction was dialysed against to tap water and finally to distilled water. The precipitate was dissolved with four to five volumes of glycerin. This glycerin solution was used as an enzyme solution. To the saturate tributylin employed as the substrate were added sodium oleate, calcium chloride, albumin, buffer solution (ammonium buffer, pH 8.5) and enzyme solution. The reaction mixture was kept at a temperature of 37°C. The activity of enzyme was measured by the falling drop method using a stalagmometer. The number of drops every 20 minutes is shown in Table 7. This quantity is approximately 0.25 butylase units. Although the enzyme

Table 7. Esterase activity of the crucian carp egg.

Ti ne	At the start	After 20 min.	After 40 min	After 60 min.
Number of drops	57	55	53	51

preparation obtained was not satisfactory good on account of the immaturity of the ovary of the crucian carp, the above results seem to show that some esterase-like substance is contained in the egg of the crucian carp.

2) *On the oil globule of the dog salmon egg*

In the study of the fertilization problem, as compared with the cortical alveoli, little attention has been paid to the oil globules. The present work deals with the general character of the oil in the dog salmon egg and the relation between the oil and protein of the egg.

In the dog salmon egg, oil globules of various sizes are found scattered throughout the yolk. But large oil globules are centered mainly at the vegetable pole, but poorly at the animal pole, except for the eggs located in the end part of the ovary. Therefore, in order to avoid the error due to lot, the egg samples used in this experiment were collected from the middle part of the ovary. Moreover, the eggs of the same external feature and of the same weight (±3 mg) were selected.

a) Measurement of the total amount of oil

The method used is very simple. Eggs were placed in mixture of 5 ml isotonic Ringer and 2g saccharose to make the egg nearly isopycnotic (1.074~1.075), and then centrifuged. In the eggs, there appeared a typical small red cap. As is well known, the red cap of centrifuged eggs consists of oil substances. By measurement of the red cap, the total amount of oil substance was determined. As shown in Fig. 1. A, the volume of the red cap was calculated by following formula.

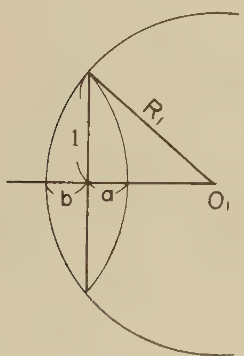


Fig. 1.A. Reference figures to introduce formula for the calculation of oil drops.

$$V = \frac{\pi}{2}\left[\frac{a^3 + b^3}{3} + 1^2 (a + b)\right]$$

where : V : the volume of the red cap,
O : the center of the egg,
1 : half length of the long axis of the red cap,
a & b : short axis of the red cap divided into a and b by long axis of the one.

The length of a, b and 1 was measured by means of horizontal microscope, and the volume of the red cap was calculated.

Foot-notes :

If the egg is considered as a sphere in shape, above calculating formula is introduced from the following formula.

As shown in Fig. 1. B, Y_1^2 and Y_2^2 are respectively expressed as follows :

$$Y_1^2 = 2\,R_1\,(b + x) - (b + x)^2 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

$$Y_2^2 = 2\,R_2\,(a - x) - (a - x)^2 \quad \ldots\ldots\ldots\ldots\ldots\ (2)$$

And then the volume of the red cap is

$$V = \pi\int_{-b}^{0} Y_1^2\,dx + \pi\int_{0}^{a} Y_2^2\,dx \quad \ldots\ldots\ldots\ldots\ (3)$$

When (1) and (2) are substituted for (3), one gets

$$V = \pi(R_1 b^2 - \frac{b^3}{3} + R_2 a^2 - \frac{a^3}{3}) \quad \ldots\ldots\ldots\ (4)$$

On the other hand, from Fig. 1.A, R_1^2 is expressed by following formula

$$R_1^2 = (R_1 - b)^2 + 1^2$$

Then one gets

$$R_1 = \frac{b^2 + 1^2}{2b} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (5)$$

Similarly one gets

$$R_2 = \frac{a^2 + 1^2}{2a} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (6)$$

When one substitutes (5) and (6) for (4), the above calculating formula is obtained.

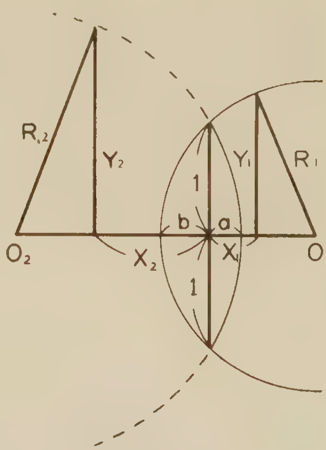
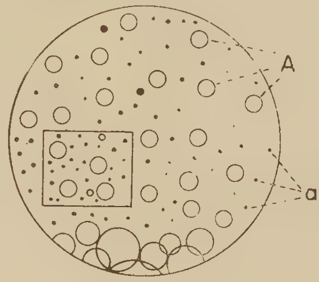


Fig. 1. B.

b) Changes in amount of egg oil with the lapse of time

It is generally accepted that the large oil globules of the dog salmon egg grow in size with the lapse of time. At first, the problem of whether the large oil globules really do grow in size or not has been investigated. The eggs were stored in refrigerator and the diameter of certain definite large oil globules were measured microscopically every day. As shown in Table 8, the large oil globules do grow in diameter. But this fact may be due to the conjugation of the minute oil granules with the large one. Then the number of oil granules (diameter below 0.005mm) in a limited area was counted with the lapse of time (Fig. 2). In fact, the oil granules decreased in number with the lapse of time (Table 9), although oil increased in total amount (Table 10). Thus the increase in diameter of the large oil globule is not always due to the conjugation of the minute oil granules.



A : oil drop
a : oil granule

Fig. 2. Distribution of oil granules and oil globules in the egg. The squared area shows the area in which the number of oil granules is counted.

Table 8. Changes in diameter of oil glouble with the lapse of time.

Experiment number / Date	1 (mm)	2 (mm)	3 (mm)	4 (mm)	5 (mm)
1 st	0.285	0.235	0.25	0.29	0.185
2 nd	0.285	0.235	0.26	0.29	0.2
3 rd	0.3	0.24	0.28	0.3	0.2
4 th	0.3	0.25	0.29	0.3	—
5 th	0.3	0.27	0.3	0.3	—

Table 9. Changes in number of oil granules in a limited area with the lapse of time.

Experiment number / Date	1	2	3	4	5	6	7	8
1 st	51	41	56	65	64	68	72	93
2 nd	45	32	53	57	60	66	66	68
3 rd	37	31	45	42	52	54	54	60
4 th	35	26	42	38	52	51	49	53
5 th	27	22	39	36	47	50	41	53
6 th	22	21	35	34	43	47	32	45

Table 10. Change in volume of oil with the lapse of time.

Date	1 st mm^3	2 nd mm^3	3 rd mm^3	4 th mm^3	5 th mm^3	6 th mm^3	7 th mm^3
Volume of oil	16.771 ±0.411	17.784 ±0.547	21.424 ±0.294	24.832 ±0.737	26.145 ±1.405	27.601 ±1.844	28.058 ±1.452

c) Effects of salts, ether, alcohol, chloreton and saponin on the total volume of egg oil

The eggs were placed in isotonic NaCl, KCl, $CaCl_2$ and $MgCl_2$ at 10°C for 3 hours and their total amount of oil was measured. As shown in Table 11, these isotonic salt solutions have no effect on the total amount of oil. As the content of the dog salmon egg is nearly isotonic with 0.95% ethyl alcohol (Takada '31), the eggs were immersed for 3 hours in 0.95% Ringer-alcohol solution and 0.95% Ringer-ether solution at 10°C, because pure isotonic alcohol solution is harmful to the egg. An increase of the total oil was detected in both series of eggs thus treated (Table 12). A similar increment of oil was also observed in eggs immersed in 0.1% saponin-Ringer solution (Table 13). On the contrary, the oil volume decreased in eggs treated with 0.5% chloreton-Ringer solution (Table 13).

Table 11. Effect of various isotonic salt (NaCl, KCl, $CaCl_2$, $MgCl_2$) solutions on the total volume of egg oil.

Salt solution	Control mm^3	Isotonic NaCl mm^3	Isotonic KCl mm^3	Isotonic $CaCl_2$ mm^3	Isotonic $MgCl_2$ mm^3
Volume of oil	16.771 ±0.411	17.564 ±0.708	17.264 ±0.985	16.988 ±0.278	17.131 ±0.346

Table 12. Effect of isotonic ether and alcohol solutions on the volume of egg oil.

Reagent solution	Control mm^3	Ether-Ringer solution (0.95%) mm^3	Alcohol-Ringer solution (0.95%) mm^3
Volume of oil	19.424 ±0.934	22.131 ±1.043	23.192 ±1.043

Table 13. Decrease and increase of oil in eggs placed respectively in 0.5% chloreton-, 0.1% saponin-Ringer solutions.

Reagent solution	Control mm^3	Isotonic Ringer mm^3	0.5% chloreton-Ringer mm^3	0.1% saponin-Ringer mm^3
Volume of oil	15.939 ±0.661	16.42 ±0.929	12.3 ±0.512	18.606 ±0.717

d) Effects of temperature, hydrogen ion concentration and ammonium chloride on total volume of egg oil

Jacob ('22) has reported that when the sea urchin eggs are exposed to a medium containg ammonium salts, the pH value of the protoplasm rises. According to Takada's data ('31), the contents of the dog salmon egg are nearly isotonic with 0.04 N HCl

and with 0.008 N NaOH respectively. Solutions of pH 5, 7 and 8 were prepared respectively by mixing 0.04 N HCl with 0.008 N NaOH. The eggs were placed in those solutions for 2 hours at 10°C, and then their total amount of oil was measured. An increase of oil volume was induced by treatment with both acid and alkaline solutions, but no appreciable change was detected in the solution of pH 7 (Table 14).

Table 14. Effect of pH on volume of oil.

pH	control mm^3	pH 5 mm^3	pH 7 mm^3	pH 8 mm^3
Volume of oil	16.792 ±0.32	24.242 ±0.372	18.891 ±0.77	21.554 ±0.825

Heilbrunn ('36) has stated that the total volume of fat in sea urchin egg is increased by immersion into NH_4Cl-sea water solution. In the dog salmon egg, the volume of oil was also increased by treatment with NH_4Cl-Ringer solution (Table 15). When the eggs were placed in Ringer-NH_4Cl solution composed of 100 ml isotonic Ringer and 5 ml M/7.5 NH_4Cl, their fertilizability was reduced up to about 50 per cent. Similarly, the fertilizability of eggs was completely lost as a result of treatment with Ringer-NH_4Cl solution containing 100 ml isotonic Ringer and 40 ml M/7.5 NH_4Cl. It is of interest that there exists a correlation between the decrease in fertilizability and increase in oil volume.

Table 15. Effect of isotonic NH_4Cl on volume of oil.

Reagent solution	Ringer only mm^3	Ringer 100ml + NH_4Cl 5ml mm^3	Ringer 100ml + NH_4Cl 40ml mm^3	NH_4Cl only mm^3
Volume of oil	16.265 ±0.34	18.857 ±0.351	18.933 ±0.533	21.899 ±1.402

Nextly, when the eggs were respectively placed in isotonic Ringer at 0°C and 10°C for 3 hours, appreciable changes in oil volumes were not visible in either series of eggs treated (Table 16). On the contrary, a marked increase in amount of oil was observed in the eggs treated with isotonic Ringer heated up to 20°C.

Table 16. Change in oil volume resultant from heating.

Temperature	0°C mm^3	10°C mm^3	20°C mm^3
Volume of oil	19.424 ±0.734	19.994 ±0.485	23.723 ±0.15

III Protein and lipoprotein at the time of activation or fertilization of the eggs of dog salmon, pond smelt and rainbow trout

1) *Protease activity of the egg and response of the cortical alveoli to both protease-activator and -inhibitor*

Mirsky ('36) has suggested that activation of sea urchin eggs is accompanied by reorganization of the egg protein. And lately, Monroy, A. and Monroy, O. A. ('51) have described the solubility changes of some protein fractions of the sea urchin egg as the result of fertilization. The protease of the egg plays an important role in the changes of protein pattern at the time of fertilization since the enzyme shows protein nature and uses itself as a tool for the splitting and syntheses of protein. The eggs and sperm proved to be interesting sources of proteolytic enzymes for the biochemist. Gustafson & Hasselberg ('51) have demonstrated a number of different enzymes in the sea urchin egg. In the same sample, the protease activity was investigated in detail by G. Lundblad ('50) and Lundblad, G, & I. Lundblad ('53).

Before going into the investigation on changes in protein pattern during early development, the present study described in this section presents a general survey of the protease system of the egg and the response of the cortical alveoli to both protease-activator and -inhibitor.

a) Protease activity of the egg

Preparation of the egg :

The eggs of the pond smelt (*Hypomesus olideus*) and the crucian carp (*Carassius auratus*) were used in this experiment. The eggs were washed repeatedly with isotonic NaCl solution at 0°C to remove ovarian fluid and then were rapidly frozen by a freezing mixture of NaCl and ice. The frozen eggs were homogenized with glass homogenizer. The homogenized eggs were extracted for three hours with five volumes of distilled water (regulated to pH 7.0) at about 0°C and centrifuged. By dialysis, the activity of the extract was largely destroyed. However, no precipitation occurred during dialysis. In this experiment, non-dialysed extract was used. The effects of media upon extraction and dilution have been tested. Isotonic NaCl and distilled water gave the same results and the minimum duration necessary for extraction was measured three hours.

The enzyme activity of the extract was tested mainly by KOH-alcohol microtitration and partly by Anson's method ('38) and formol titration. The maximum value has been designated as 100, because the present study aims to determine the comparative activity of the same enzyme activity in different developmental stages.

b) Activation and inhibition of protease of the egg

Although the trypsin and pepsin type enzymes do not require any activator for the manifestation of their activity, papain and cathepsin type enzymes require activators such as cyanide, ascorbic acid, cysteine and gluthathion, and also the activity of metal protein (for example, metal peptidase) is greatly enhanced by the presence of metal ions (Smith '51).

In the present experiments the effects of various protease activators and -inhibitors on the enzyme activity of eggs were investigated. Of these activators, cysteine, at pH 6.2, did not affect the activity of enzyme. Similarly, hydrocyanide showed no desirable effect on the activation of proteinase (gelatin-splitting enzyme) of the egg. This enzyme became inactivated by the addition of mercury bichloride and was again restored by hydrocyanide (Table 17). On the other hand, the inhibitory action of mercury bichloride could not be overcome by the addition of cysteine.

Table 17. Changes in proteolytic activity of the pond smelt egg caused by inhibitor and activator.

Experiment number	Reagent added to the reaction mixture*	Relative per cent of enzyme activity
1	activity of unfertilized egg (control)	100
2	0.1 ml of 10^{-3}M mercury bichloride	35
3	0.1 ml of 10^{-3}M hydrocyanide	93
4	0.1 ml of 10^{-3}M mercury bichloride～ 0.2 ml of 10^{-3}M hydrocyanide	80
5	0.1 ml of 10^{-3}M mercury bichloride～ 0.2 ml of 10^{-3}M cysteine	33

* The reaction mixture consisted of 1 ml of 0.1 % gelatin, 0.5 ml of veronal buffer (pH 6.9) and 0.2 ml of enzyme solution, and was incubated at 30°C for 2 hrs. To a blank test, same volume of distilled water instead of the extract was added, and both experiment and control were run simultaneously.

Exp. No. 4 : 0.1 ml of 10^{-3}M mercury bichloride was added to the reaction mixture and then 0.2 ml of 10^{-3}M hydrocyanide was added 2 hrs. after the start. The activity was measured 2 hrs. later (total 4 hrs.), and

Exp. No. 5 : 0.1 ml of 10^{-3}M mercury bichloride was adedd to the reaction mixture and the 0.2 ml of 10^{-3}M cysteine added 2 hrs. after the start under anaerobic condition by means of Thunberg's tube. The activity was measured 2 hrs. after (total 4 hrs.).

Glycyl-glycine dipeptidase was found in egg of the crucian carp. It is generally accepted that metal ions required for activation are highly specific for dipeptidase of different tissues. The glycyl-glycine dipeptidase from the egg of the crucian carp was apparently activated by Mn^{++} ions, but the Co^{++} ion which is a common activator of glycyl-glycine dipeptidase showed no effects on the activity of the enzyme. Ca^{++} and Mg^{++} ions acted on the enzyme as inhibitors. The fatty acid is also known as an inhibitor of peptidase. The glycyl-glycine dipeptidase of the egg was also inactivated by fatty acids such as butyric and succinic acids. These results are summarized in Table 18.

Table 18. Changes in peptidase activity of the crucian carp egg caused by metal ions and fatty acid.

Reagent added to the reaction mixture*	Relative per cent of enzyme activity
Activity of unfertilized egg (control)	100
0.2 ml of 10^{-3}M $MnCl_2$	174
0.2 ml of 10^{-4}M $MnCl_2$	138
0.2 ml of 10^{-3}M $CaCl_2$	60
0.3 ml of 10^{-3}M $CaCl_2$	56
0.1 ml of 10^{-3}M $MgCl_2$	60
0.2 ml of 10^{-3}M $MgCl_2$	55
0.1 ml of M/20 butylic acid	83
0.1 ml of M/20 succinic acid	58
0.2 ml of M/20 succinic acid	87

* The reaction mixture consisted of 1 ml of 0.1 % glycyl-glycine, 0.5 ml of veronal buffer (pH 8.2) and 0.2 ml of enzyme solution, and was incubated at 30°C for 2 hrs. To a blank test, the same volume of distilled water was added instead of the extract, and both experiment and control were run simultaneously.

From the data it may be said that the papain and cathepsin type enzymes and especially metal peptidase are present in the eggs. Generally, enzyme activity of the egg was very weak but the glycyl-glycine dipeptidase showed stronger activity than proteinase.

c) The effects of protease-activators and -inhibitors on the egg of the pond smelt

Since the presence of some protease system was confirmed in the eggs, nextly, the responses of protease-activator and -inhibitor on the eggs were investigated. As the inhibitors of enzymes, monoiodo-acetate, mercury bichloride, lead-acetate and potassium-iodide were employed. On the other hand, cysteine, ascorbic acid and hydrocyanide were used as activators. The unfertilized eggs were put into the Ringer-activator solution for 10 minutes and then washed with isotonic Ringer to remove protease-activator. After these treatments, the fertilization rate and activation of the eggs were examined. In the second experiment, after treatment with Ringer-inhibitor solution, the eggs were transferred to Ringer-activator solution and treated for 10 minutes, then the fertilization rate was observed. In the third experiment, the eggs were placed in Ringer-inhibitor solution and then replaced in another Ringer-inhibitor solution for 10 minutes. Also, the eggs were similarly treated twice with Ringer-activator solution. The effects of these treatments upon fertilization capacity were observed. As the blank test, the effects of these reagents on the spermatozoa were investigated. The results are shown in Table 19. Among the various enzyme inhibitors used, mercury bichloride gave harmful effect on fertilization reaction. But this harmful effect of mercury bichloride on the egg could be overcome by treat-

Table 19. Influence of protease-activator and -inhibitor upon activation or fertilization of pond smelt eggs.

Ringer-activator (or inhibitor) solution used in treatments of the eggs	Blank test on sperm	Activation (%)	Fertilization (%)	Remarks
Control			99	
10^{-4} M Ringer-monoiodo-acetate	no change	no activation	78-80	Treatment with Ringer-inhibitor
10^{-4} M Ringer-mercury bichloride	some harmful effect		0	
10^{-4} M Ringer-lead-acetate	some harmful effect		50-60	
10^{-4} M Ringer-lead-phosphate	no change		85-90	
10^{-3} M Ringer-potassium-iodide	no change		90<	
10^{-4} M Ringer-hydrocyanide	no change		85-90	Treatment with Ringer-activator
10^{-3} M Ringer-cysteine	no change		85-90	
10^{-3} M Ringer-ascorbic acid	no change		90<	
10^{-4} M Ringer-mercury bichloride $\sim 10^{-3}$ M Ringer-cysteine		90	5-8	Treated with Ringer-inhibitor and then transferred to Ringer-activator
10^{-4} M Ringer-mercury bichloride $\sim 10^{-4}$ M Ringer-hydrocyanide		no activation	82-85	
10^{-4} M Ringer-lead-acetate $\sim 10^{-3}$ M Ringer-cysteine			70-75	
10^{-4} M Ringer-lead-acetate $\sim 10^{-4}$ M Ringer-hydrocyanide			75-80	
10^{-4} M Ringer-monoiodo-acetate $\sim 10^{-3}$ M Ringer-cysteine		50-60	25-30	
10^{-4} M Ringer-monoiodo-acetate $\sim 10^{-4}$ M Ringer-hydrocyanide		30-40	50	
10^{-3} M Ringer-potassium-iodide $\sim 10^{-3}$ M Ringer-cysteine		100		
10^{-3} M Ringer-potassium-iodide $\sim 10^{-4}$ M Ringer-hydrocyanide		100		
10^{-4} M Ringer-mercury bichloride $\sim 10^{-3}$ M Ringer-ascorbic acid		100		
10^{-4} M Ringer-monoiodo-acetate $\sim 10^{-4}$ M Ringer-lead-acetate		no activation		Exposed to Ringer-inhibitor and then retreated with another species of Ringer-inhibitor
10^{-4} M Ringer-mercury bichloride $\sim 10^{-3}$ M Ringer-potassium-iodide		50<		
10^{-3} M Ringer-potassium-iodide $\sim 10^{-4}$ M Ringer-monoiodo-acetate		no activation		
10^{-3} M Ringer-cysteine $\sim 10^{-3}$ M Ringer-ascorbic acid		no activation	90<	Exposed to Ringer-activator and then retreated with another species of Ringer-activator

ment with hydrocyanide. Ringer-lead-acetate showed also activating effect on the egg, as has already been described in this paper (Chapter II), that is, in the crucian carp eggs the breakdown of the cortical alveoli is caused by treatment with Ringer-lead-acetate solution. The activating effect of lead-acetate on the egg was also overcome in part by hydrocyanide.

2) *Electrophoretic studies of protein and protein fractions in activation and fertilization of the eggs*

A large number of biochemical studies on activation and fertilization of sea urchin eggs have been made, mostly from the metabolic standpoint. However, little attention has been paid to the changes in protein pattern. These changes were firstly reported by Mirsky ('36) who demonstrated that KCl soluble protein fraction of the unfertilized sea urchin egg becomes insoluble as a result of fertilization. These rearrangement of the protein pattern are suggested by observations concerning the changes of viscosity, permeability and cortical layer etc. which occurred in activated egg. As compared with the studies on sea urchin egg, those on fish eggs are too few. The activating reaction is the first step of the changes which take place in the process of fertilization. The fertilization reaction is brought about by two necessary factors, viz., the entry of the spermatozoa and the activation of the egg itself. In contact with water, the unfertilized eggs of the fresh water fish are easily activated without insemination. Simultaneously, the eggs undergo certain cortical changes and absorption of water (Aoki '39, Kanoh '50). The morphological change of the activated eggs caused either by water or by the normal fertilization is the same until the egg begins to cleave. That is to say, there occur the same cortical changes and the subsequent elevation of the chorion.

In the present study the difference between activated and fertilized eggs has been investigated by electrophoresis and the changes in protein pattern at the time of activation were subjected to physicochemical analysis.

a) Preparation of the egg:

As the materials of this work, the eggs of the pond smelt (*Hypomesus olideus*), dog salmon (*Oncorhynchus keta*) and rainbow trout (*Salmo irideus*) were employed. The eggs were washed repeatedly in isotonic NaCl solution (M/7.5) at 0°C to remove ovarian fluid, broken by pressure, and then centrifuged in order to separate yolky substance from the free lipid and egg-membrane (chorion) as completely as possible. In this case, the yolky fraction (whole yolk) probably includes all substances other than the oil drops of the egg. After separation of the supernatant oil and chorion, the whole yolk was diluted with 20 volumes of phosphate buffer solution (pH 7.0), and was left to dialyzed for about 48 hours at 4°C. After dialysis, the sample was submitted to an electrophoretic analysis. Another electrophoretic sample was prepared as follows: Above whole yolk was diluted with an equal volume of 10% NaCl, adjusted to pH 7.5. The diluted yolk then was extracted 5 to 10 times at 0°C with ethyl ether free from peroxide in a separatory funnel, until the lipid in ether extracts became negligible. The ether extracted yolk was diluted with 10 volumes of phosphate buffer solution (pH 7.0), and dialysed. It was then subjected to electrophoretic

analysis which was carried out with HT-B type Tiselius apparatus with size of cell $2\times15\times$ 50 mm. All of the experiments were carried out in Na_2HPO_4-KH_2PO_4 buffer (pH 7.0), with ionic strength approximately 0.3.

b) Electrophoretic difference between activated and fertilized eggs of the pond smelt

The whole yolk of unfertilized (as a control), fertilized and activated (by water) eggs were respectively submitted to the electrophoresis. The yolk fractions of fertilized eggs were obtained from the eggs 2 minutes and 15 minutes after the fertiliza-

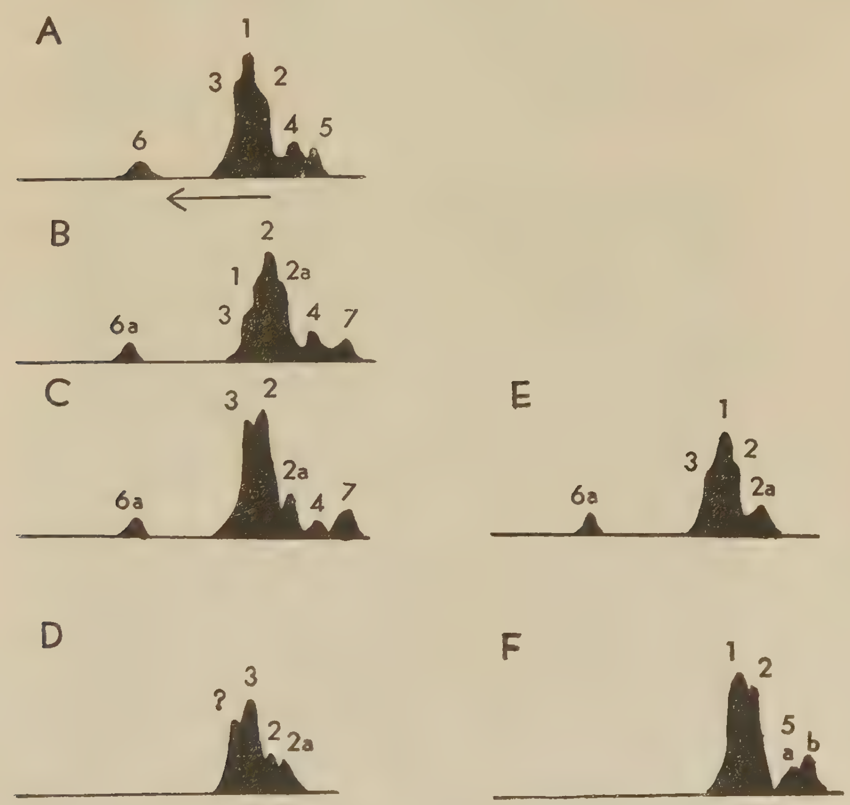


Fig. 3. Electrophoretic diagrams of whole yolk of *Hypomesus olidus*.
A) unfertilized eggs; B) eggs 30 seconds after activation; C) eggs 2 minutes after activation; D) eggs 15 minutes after activation; E) eggs 2 minutes after fertilization; F) eggs 15 minutes after fertilization.
Electrophoresis in pH 7.0; potential grade, A,B,D,E and F=7.3 volt/cm., C=7.4 volt/cm. Period of electrophoresis, A,B,C,E and F=3650 sec., D=3606 sec. ?=uncertain presence of a component. ←=direction of migration.
(Representation of the components has been prepared by tracing the original photographs. The diagram shows the descending limb.)

tion, and those of activated eggs were obtained 30 seconds, 2 minutes and 15 minutes after immersing into water. The above stated times were determined in consideration of fertilizability of the eggs. That is to say, the fertilization rate of the egg is reduced to about 50% by a 30 seconds' immersion into water and to zero by 2 minutes' immersion. In the electrophoretic diagram of the whole yolk of unfertilized eggs, six components were recognized (Fig. 3. A). As shown in Fig. 3, in case of activated eggs, component No. 1 disappeared after 30 seconds, while components No. 2 and No. 3 did not disappear for 15 minutes after activation (Fig. 3, B, C and D). Component No. 4 disappeared 2 minutes after activation, and 30 seconds later component No. 5 was not to be seen. No. 6, the fastest component, became even faster as the result of activation and disappeared 2 minutes later. Component No. 6a may be derived from component No. 6 of unfertilized egg. The new component No. 2a did not disap-

Table 20. Changes in mobility (μ. 10^{-5} cm^2/volt. sec.) and relative ratio of protein components of whole yolk of pond smelt eggs at the time of activation and of fertilization.

Component Number	Component Mobility and Ratio(%	Unfertilized eggs	30 seconds after activation	2 minutes after activation	15 minutes after activation	2 minutes after fertilization	15 minutes after fertilization
?	M* R*				5.1 24.1		
1	M R	3.9 28.5	3.9 11.0			4.0 39.1	3.7 42.9
2	M R	3.1 13.5	3.4 27.7	3.2 47.1	3.2 14.7	3.4 22.7	3.1 37.6
2a	M R		2.7 26.5	2.4 7.8	2.4 13.8	2.4 9.1	
3	M R	4.3 23.7	4.3 10.3	4.3 31.3	4.3 47.4	4.4 21.8	
4	M R	1.3 14.4	1.3 11.6	1.3 3.1			
5	M R	0.9 9.1					a M 0.9, R 8.1 b M 0.6, R 11.4
6	M R	8.9 13.5					
6a	M R		10.3 6.4	10.3 3.8		10.8 7.3	
7	M R		+0.5 6.5	+0.5 6.9			

* M : Mobility R : Ratio(%) "Component" represents those of the descending limb.

pear for 15 minutes after activation. In front of component No. 3 a faster moving component was present (Fig. 3. D. ?). This component must also be a new one. Further, it is noticeable that the opposite charged component No. 7 appears as the result of activation. On the other hand, in fertilized eggs, components No. 1 and

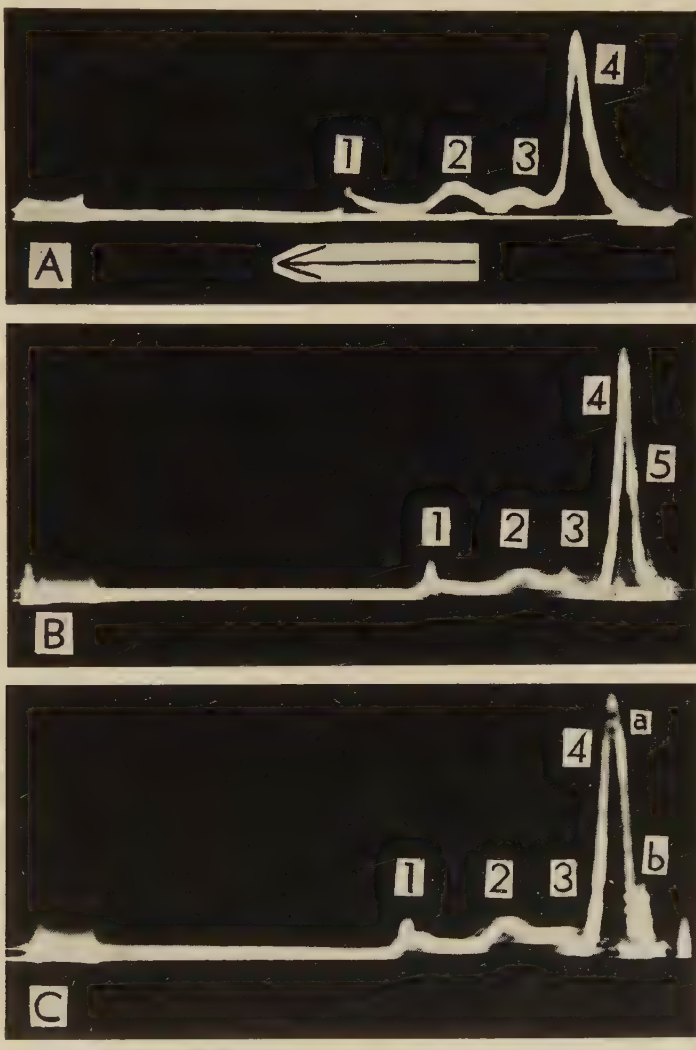
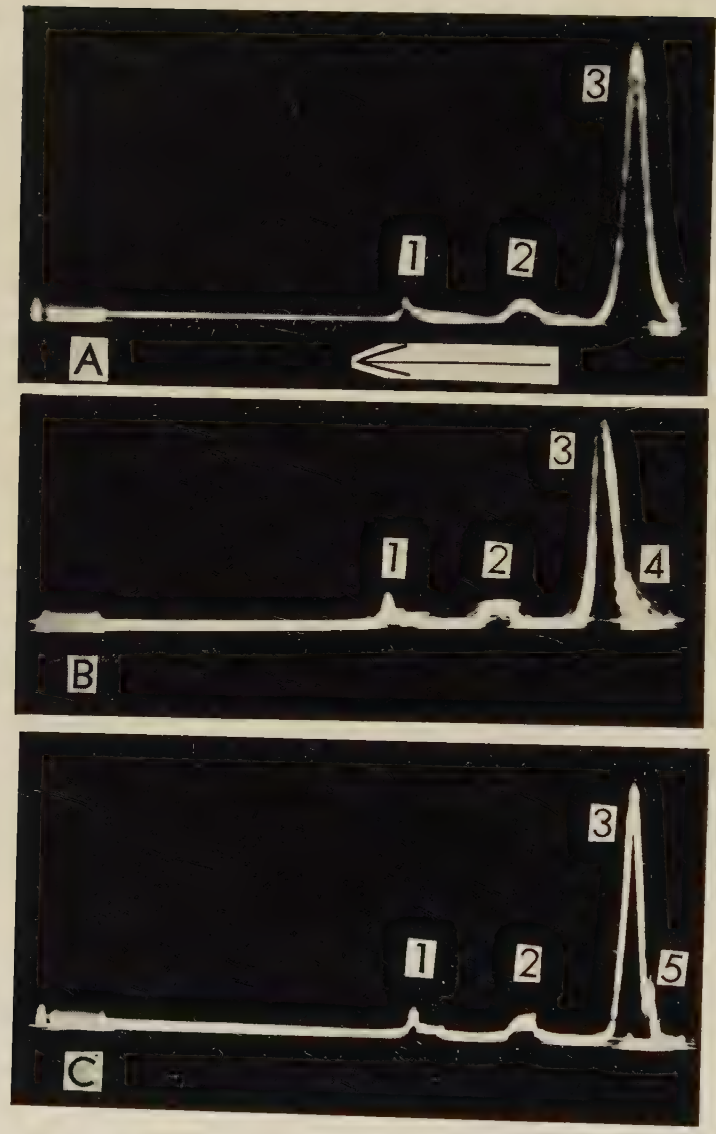


Fig. 4. Electrophoretic diagrams of whole yolk of *Oncorhynchus keta*.
A) unfertilized eggs; B) eggs 2 minutes after activation; C) eggs 15 minutes after activation.
Electrophoresis in pH 7.0; potential grade, A=5.7volt/cm., B and C=5.8 volt/cm. after 3600 sec. ←=direction of migration.
(The diagram shows the descending limb.)



No. 2 did not disappear for 15 minutes after fertilization, but components No. 3 and No. 2a disappeared 2 minutes later (Fig. 3. E and F). Component No. 5b appeared 15 minutes after fertilization (Fig. 3. F). This component is considered to be a new one. The opposite charged component was not visible in fertilized eggs. From the above findings, it may be said that the components of the whole yolk diminished in number during 2 minutes after activation or fertilization. During this time, activated eggs lose fertilizability completely. These facts show that some great changes

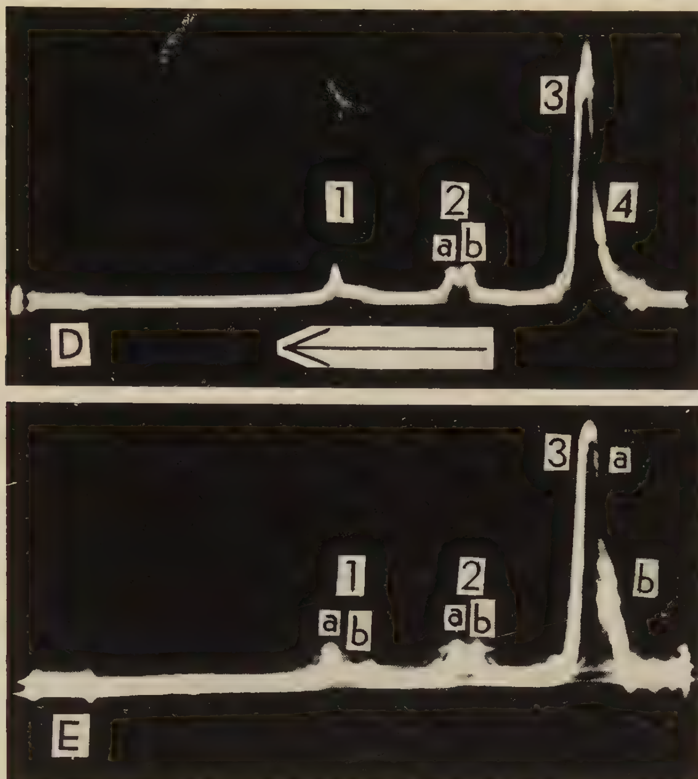


Fig. 5. Electrophoretic diagrams of ether extracted yolk of *Oncorhynchus keta*. A) unfertilized eggs; B) eggs 2 minutes after activation; C) eggs 15 minutes after activation; D) eggs 2 minutes after fertilization; E) eggs 15 minutes after fertilization. Electrophoresis in pH 7.0; potential grade, A and C=5.7 volt/cm., B=5.6 volt/cm., D=6.7 volt/cm. and E=7.0 volt/cm. after 3600 sec. ←=direction of migration.
(The diagram shows the descending limb.)

occur in the egg during 2 minutes after fertilization (or activation). Of these components which disappeared within 2 minutes, components No. 6a and No. 7 probably play an important part in the activating process. Thus, the electrophoretic diagram in fertilized egg resembles generally that of the activated egg, but the mobility of each component is different (Table 20). Therefore, the two kinds of eggs, activated and fertilized, have a different quality of protein level respectively.

c) Electrophoretic analysis in the dog salmon and the rainbow trout eggs

In the previous section, the writer has reported the electrophoretic difference between activated and fertilized eggs in the pond smelt. The same procedure was

applied to prepared the whole yolk of dog salmon and rainbow trout. The ether extracted yolk prepared from the unfertilized (as a control), activated and fertilized eggs of the two kinds of fishes were subjected to an electrophoretic analysis. In consideration of fertilizability, activated eggs used as the materials were treated for 2 and 15 minutes with water respectively, because the fertilization rate of the egg was decreased to 50% in 2 minutes and reduced to zero in 15 minutes after immersing in water. Fertilized eggs, 2 and 15 minutes after fertilization, were employed as the material for comparison with the activated eggs. In the electrophoretic diagram of whole yolk of unfertilized eggs of the dog salmon, four components were recognizable (Fig. 4). But in the ether extracted yolk of unfertilized egg, only three components were detected (Fig. 5). Component No. 3 of whole yolk disappeared in the process of ether extraction. But it seems improbable that this component plays any important part in the activating processes. The opposite charged component appeared in 2 minutes after activation while it was not visible in the fertilized egg. In ether extracted yolk of fertilized egg, all components have been divided into two components at 15 minutes after fertilization. As shown in Table 21, all the components of whole yolk decrease in mobility within 2 minutes after activation, but recover their mobility 15 minutes later. On the contrary, the components of ether extracted yolk increase in mobility in 2 minutes after activation and recover their mobility 15 minutes later. In fertilized egg, the mobility of components decreased 15 minutes later (Table 22).

Table 21. Changes in mobility (μ. $10^{-5}cm^2/volt.\ sec.$) and relative ratio of protein components of whole yolk of dog salmon eggs at the time of activation.

Component: Number	Component: Mobility and Ratio (%)	Unfertilized eggs	2 minutes after activation	15 minutes after activation		
1	M*	8.7	7.4			8.4
	R*	11.8	12.2			10.2
2	M	4.1	3.8			4.8
	R	18.2	15.3			12.4
3	M	2.8	2.3			3.1
	R	10.0	8.2			8.7
4	M	0.6	0.3	a	M	1.1
					R	60.0
	R	60.0	46.0	b	M	0.2
					R	8.7
5	M		+0.2			
	R		8.3			

* M : Mobility R : Ratio (%) "Component" represents those of the descending limb.

Table 22. Changes in mobility (u. $10^{-5}cm^2$/volt. sec.) and relative ratio of protein components of ether extracted yolk of dog salmon eggs at the time of activation and of fertilization.

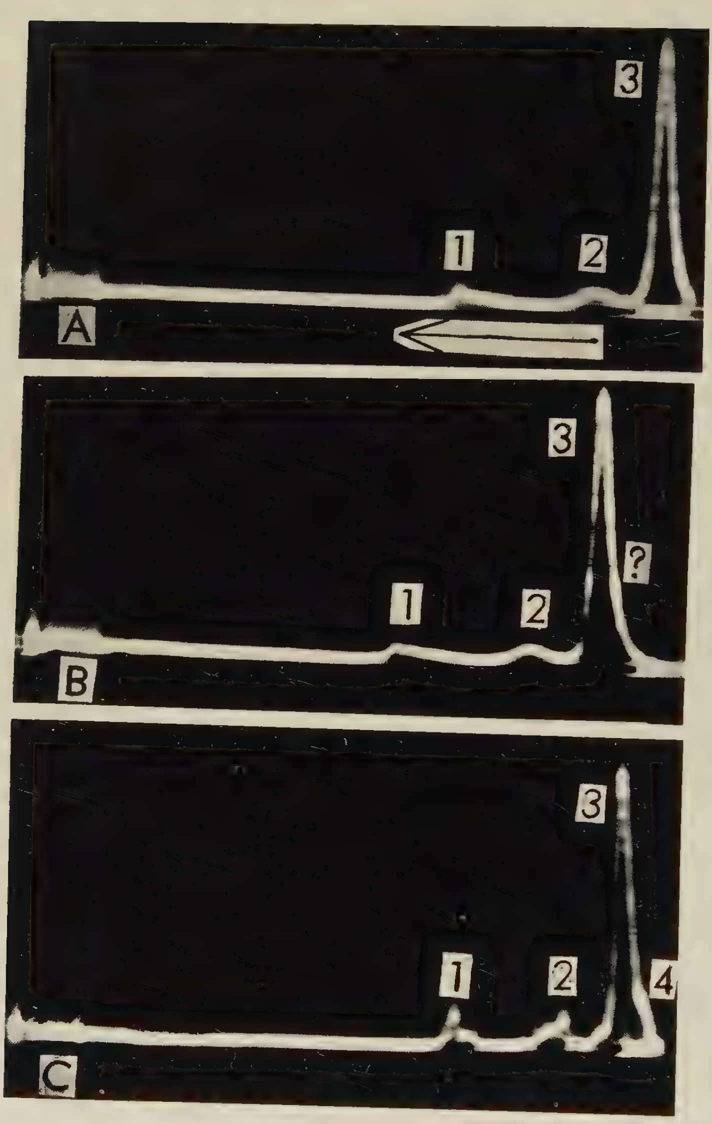
Component Number	Mobility and Ratio (%)	Unfertilized eggs	2 minutes after activation	15 minutes after activation	2 minutes after fertilization	15 minutes after fertilization
1	M*	8.7	9.1	8.5	8.3	a: M 8.5, R 13.0
	R*	9.8	11.7	12.6	14.0	b: M 7.5, R 5.5
2	M	4.6	4.9	4.3	a: M 4.5, R 7.7	4.9, 10.0
	R	10.2	11.4	10.0	b: M 4.0, R 7.0	4.4, 6.4
3	M	1.0	0.9	0.5	0.5	a: M 1.0, R 54.8
	R	80.0	67.2	70.4	57.0	b: M 0.5, R 10.3
4	M		0.2		0.2	
	R		9.7		14.3	
5	M			+0.4		
	R			7.6		

* M : Mobility R : Ratio(%) "Component" represents those of the descending limb.

Table 23. Changes in mobility (μ. $10^{-5}cm^2$/volt. sec.) and relative ratio of protein components of ether extracted yolk of rainbow trout eggs at the time of activation and of fertilization.

Component Number	Mobility and Ratio (%)	Unfertilized eggs	2 minutes after activation	15 minutes after activation	2 minutes after fertilization	15 minutes after fertilization
1	M*	8.3	7.3	8.6	7.6	9.2
	R*	13.8	13.4	10.5	14.5	16.3
2	M	3.2	2.4	3.1	2.6	4.1
	R	14.7	12.3	9.5	12.7	14.6
3	M	0.7	0.6	1.0	0.4	2.0
	R	71.5	62.0	73.6	72.8	69.1
4	M			+ 0.5		
	R			6.4		
?	M		+ 0.2			
	R		12.3			

* M : Mobility R : Ratio (%) "Component" represents those of the descending limb.



The ether extracted yolk of the rainbow trout eggs also showed the same electrophoretic figure as those of the dog salmon egg. For example, the opposite charged component appeared in 2 minutes after activation while this component was not visible in fertilized egg. The changes in electrophoretic diagrams at the time of activation and of fertilization are shown in Fig. 6. All the components of ether extracted yolk decreased in mobility as the result of activation or fertilization. In fertilized eggs, the components increased in mobility again 15 minutes later (Table 23).

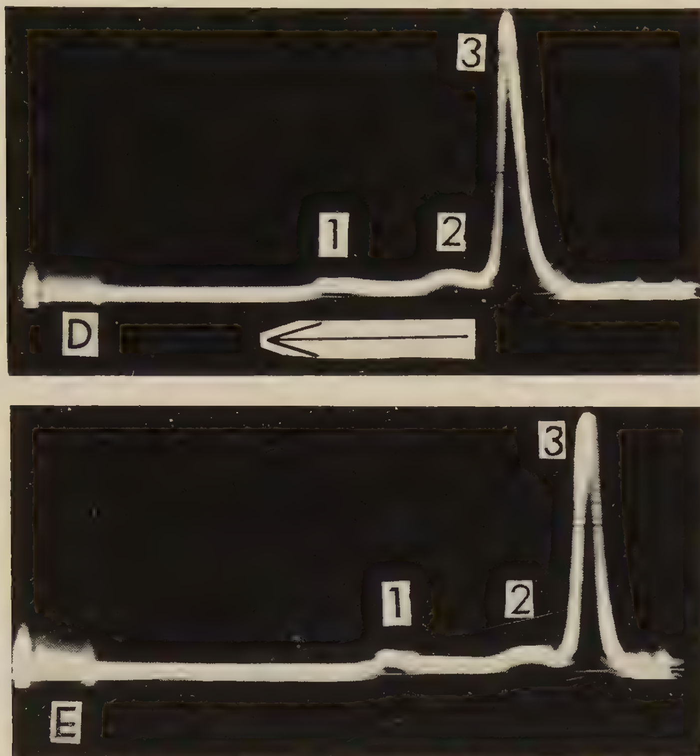


Fig. 6. Electrophoretic diagrams of ether extracted yolk of *Salmo irideus*.
A) unfertilized eggs; B) eggs 2 minutes after activation; C) eggs 15 minutes after activation; D) eggs 2 minutes after fertilization; E) eggs 15 minutes after fertilization.
Electrophoresis in pH 7.0; potential grade, A=5.8 volt/cm., B=5.9 volt/cm., C=5.6 volt/cm., D=5.4 volt/cm. and E=5.5 volt/cm. after 3600 sec ←=direction of migration. ? =uncertain presence of a component.
(The diagram shows the descending limb.)

3) *Denaturation of egg protein at the time of activation of the egg*

The denaturation of egg protein at the time of activation has already been ascertained by electrophoretic analysis. Evidently, denaturation is a complex phenomenon and our knowledge about the mechanism of denaturation is still incomplete. The phenomenon of denaturation has been explained in a variety of ways. Further, in order to ascertain whether the denaturation of egg protein really occurs at the time of activation, the following experiments were carried out. In the first experiment, the electrophoretic diagram of activated egg was compared with that of unfertilized egg denaturated by heat. In the second experiment, color reaction was examined with

the lapse of time after activation of the eggs.

On the other hand, the appearance of opposite charged component at the time of activation has already been shown by electrophoretic analysis. This may be due to the interaction between each of the protein fractions of eggs. And also, it seems probable that this component was caused to appear by the lysozyme-like substance of the egg as demonstrated in the hen's egg (Fevold '51). In order to ascertain this point, the interaction between water soluble protein and levetin fractions of egg protein has been studied and further the presence of lysozyme-like substance in the eggs was examined. Moreover, some experiments concerning the fractionation of egg protein of rainbow trout were also performed.

a) Protein-protein interaction

In the egg white of the hen's egg, the opposite charged component makes appearance due to the interaction between ovomucoid and globulin (Longsworth *et al.* '40). The appearance of this component at the time of activation may also be due to the interaction between these proteins. The unfertilized eggs of the dog salmon were broken by pressure, and then centrifuged to remove the supernatant oil and chorion. An equal volume of water (pH 7.0) was added to the yolk (whole yolk) and it was centrifuged to separate the water soluble fraction. On the other hand, levetin-like fraction was prepared from the whole yolk by dilution with 10 volumes of water adjusted to pH 5.0. The resultant precipitate was redissolved in 10% NaCl. This levetin-like fraction and the water soluble fraction were mixed and diluted with phosphate buffer. This sample was subjected to an electrophoresis after dialysis. But in the electrophoretic diagram of this sample, the opposite charged component did not appear. In general, when protein concentration of the sample is high and its ionic strength is low, the opposite charged component tends to appear (Longsworth *et. al.* '40). But in the present experiments, the protein concentration was below 1% and ionic strength was approximately 0.3.

b) Confirmation of lysozyme-like substance

In the electrophoretic diagram of lysozyme of the hen's egg, the opposite charged component appeared (Fevold '50). By means of Alderton, Ward and Fevold's method ('45), the writer attempted to prepar the lysozyme-like substance from the egg of dog salmon. But no satisfactory result was obtained in this experiment.

c) Fractionation of egg protein of the rainbow trout

Young and Phinney ('51) fractionated yolk protein of the Atlantic salmon (*Salmo salar*). Their preparation exhibited three components as the result of electrophoretic analysis. They interpreted the diagram to indicate lipovitellin, levetin and vitello-

mucoid or phosvitin. The writer tried to fractionate the yolk protein of the rainbow trout electrophoretically. Although the fractions obtained by the present method did not show desirable degree of homogeneity, it seems worthwhile to describe the results. The method of preparation is based mainly on Young and Phinney's ('51). As stated above, the ether extarcted yolk from the unfertilized eggs had been stored as a stock solution at 0°C. The fraction of protein was prepared from this stock solution. Lipoprotein was prepared from the stock solution by isoelectric precipitation. After two times reprecipita ion, the resulting precipitate became insoluble in buffer solution. The precipitate was redispersed in 10% NaCl, diluted with buffer solution and then examined electrophoretically after dialysis. Nevertheless, the sample did not show characteristic diagram. Considerable denaturation necessarily occurred in process of preparation.

Fractionation of lipovitellin gave good results in this experiment. Lipovitellin was prepared by dialysis of the stock solution, essentially by the procedure of Chargaff ('42). A portion of the final precipitate in the moist state was dissolved in a buffer solution and examined electrophoretically. But there were always three boundaries, even after four reprecipitations. Young and Phinney ('51) have reported that a single boundary was obtained for hen lipovitellin, but there were always three boundaries in the case of the Atlantic salmon (*Salmo salar*). In consideration of their results, component No. 3, shown in Fig. 7. A, must be a lipovitellin fraction. Their mobilities are shown in Table 24. From the above fact, the opposite charged component which appears at the time of activation is considered to be derived from lipovitellin fraction.

Levetin was also prepared from stock solution by dilution with 10 volumes of water adjusted to pH 5.0. The centrifuged precipitate was redissolved in 10% NaCl with some difficulty. The dilution, precipitation and redissolution of the sample were similarly repeated once more, and the sample thus prepared was examined electrophoretically. But the sample did not show a characteristic figure. Thus, although only lipovitellin fraction could not be demonstrated electrophoretically, the electrophoretic diagram of ether extracted yolk of the rainbow trout is quite similar to that of Atlantic salmon. Therefore, three migrating boundaries of the ether extracted yolk of rainbow trout may also indicate lipovitellin, levetin and phosvitin (or vitellomucoid) as in the case of Atlantic salmon eggs.

d) Denaturation of protein fraction by heat

From the fact that the opposite charged component appeared in a lipovitellin fraction of egg protein, the writer assumed that denaturation of egg protein is the first step of chemical change at the time of activation. If that is the case, the electrophoretic pattern of the activated egg must be considerably similar to that of unfertilized egg denaturated by heat. According to Young and Phinney ('51), lipovitellin appears to be readily denaturated in contact with water at 20°C. Lipovitellin fraction

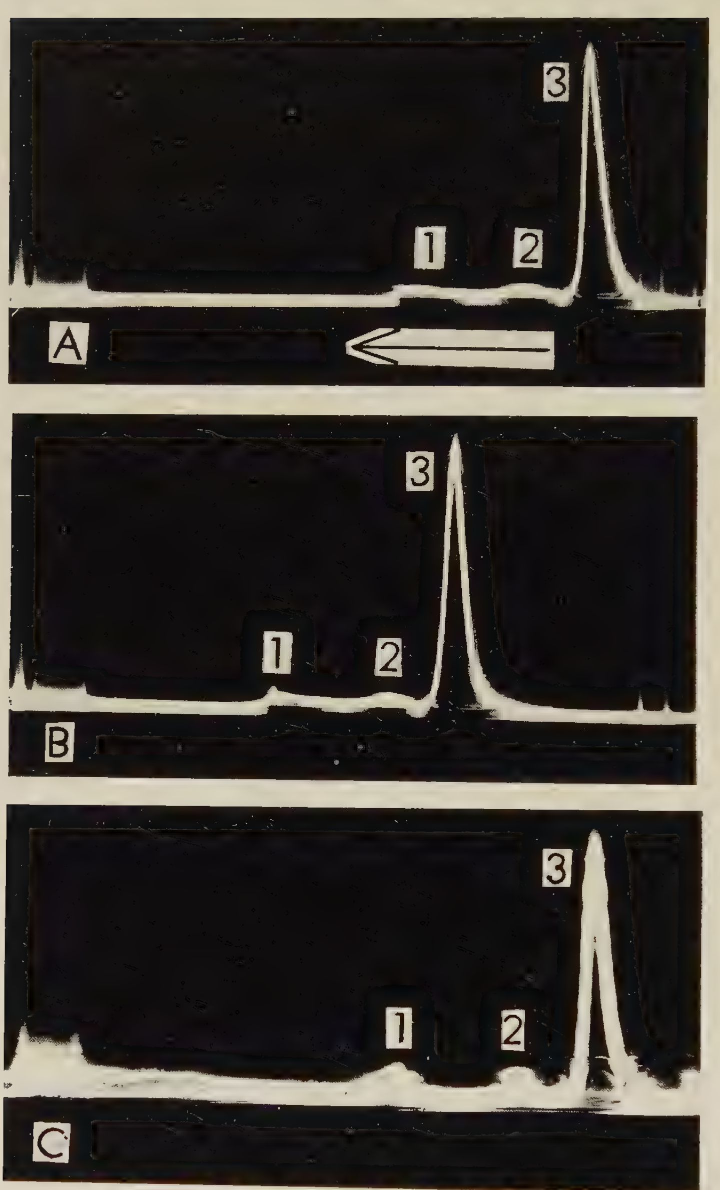


Fig. 7. Electrophoretic diagrams of lipovitellin fraction and heat denaturated fraction of rainbow trout eggs.

A : non-treated (control) lipovitellin fraction,

B : lipovitellin fraction denaturated at 50°C for 10 min. and

C : lipovitellin fraction denaturated at 30°C for 30 min.

Electrophoresis in pH 7.0, potential grade, A and B=5.5 volt/cm. and C=5.8 volt/cm. after 3600 sec. ←=direction of migration.

(The diagram shows the descending limb.)

Table 24. Changes in mobility (μ. $10^{-5}cm^2$/volt. sec.) and relative ratio of heated lipovitellin fraction.

Component Number	Mobility and Ratio (%)	Lipovitellin fraction (non-denaturated)	Denaturation of lipovitellin at 30°C for 30 minutes	Denaturation of lipovitellin at 50°C for 10 minutes
1	M*	7.1	7.1	10.0
	R*	13.7	13.9	14.8
2	M	3.1	2.9	5.9
	R	12.6	13.9	12.8
3	M	0.8	0.3	3.6
	R	73.9	72.2	72.4

* M : Mobility R : Ratio (%)

prepared from the unfertilized rainbow trout eggs was denaturated at 30°C for 30 minutes and 50°C for 10 minutes. During these procedures, no coagulation of the samples occurred. After these treatments, the samples were subjected to an electrophoretic analysis. The changes in electrophoretic diagrams of lipovitellin fraction from unfertilized egg yolk as a result of heating are shown in Fig. 7. As shown in Table 24, the mobility of lipovitellin fraction is decreased by treatment at 30°C for 30 minutes, but increased by treatment at 50°C for 10 minutes. In fact, the movement of lipovitellin fraction become faster at the time of fertilization and slower at activation. The opposite charged component did not appear in these cases.

Similarly, ether extracted yolk from the unfertilized eggs of rainbow trout was heated at 50°C for 15 minutes after equal volume of water had been added, while another sampe was heated at 50°C for 30 minutes without addition of water. These samples were examined electrophoretically. By 15 minute treatment, components of egg protein decreased in mobility, while components treated for 30 minutes increased in mobility (Fig. 8 and Table 25). In this case, the opposite charged component did not appear as in the case of denaturation of lipovitellin fraction by heat. From the above results, it seems certain that a weak degree of protein denaturation occurs at the time of activation and fertilization.

e) Changes in color reaction caused by activation of the egg

Denaturation of protein is indicated by the color reaction which is given more intensively by denaturated protein than by the same protein in the native state. Here, the changes in color given by biuret reaction were spectrophotometrically examined with the lapse of time after activation. The measurement was carried out according to Araya ('49). The results as shown in Fig. 9 indicated that color reaction becomes more intensive with the lapse of time after activation or fertilization. As stated

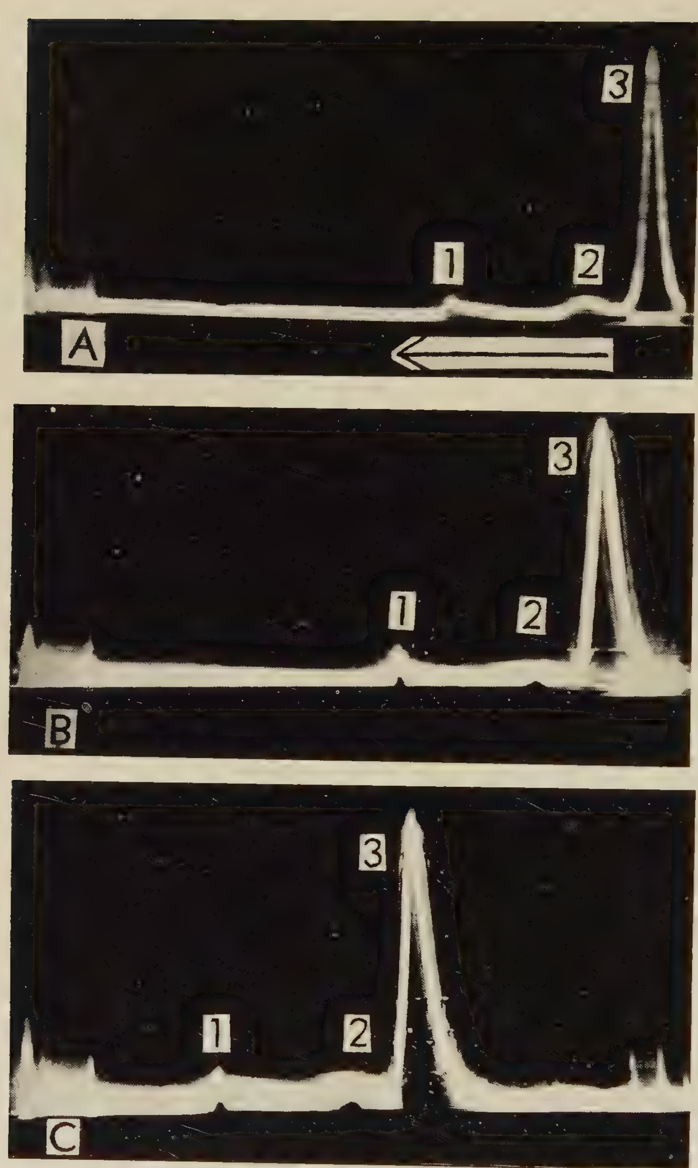


Fig. 8. Electrophoretic diagrams of ether extracted yolk and heat denaturated yolk of rainbow trout eggs.

A : non-treated (control) ether extracted yolk of unfertilized eggs,

B : equal volume of water added to the ether extracted yolk of unfertilized eggs and denaturated at 50°C for 15 min., and

C : ether extracted yolk of unfertilized eggs denaturated at 50°C for 30 min. without addition of water.

Electrophoresis in pH 7.0, potential grade, A=5.8 volt/cm., B=5.1 volt/cm. and C=5.4 volt/cm. after 3600 sec. ←=direction of migration. (The diagram shows the descending limb.)

Table 25. Changes in mobility (μ. $10^{-5}cm^2$/volt. sec.) and relative ratio of protein components of heated ether extracted yolk.

Component: Number	Component: Mobility and Ratio (%)	Ether extracted yolk of unfertilized eggs (non-denaturated)	Denaturated at 50°C for 30 minutes without water	Denaturated at 50°C for 15 minutes with equal volume of water
1	M*	8.3	14.2	7.6
	R*	13.8	10.3	10.0
2	M	3.2	9.5	2.3
	R	14.7	9.2	12.3
3	M	0.7	6.8	0.8
	R	71.5	80.5	77.7

* M : Mobility R : Ratio (%)

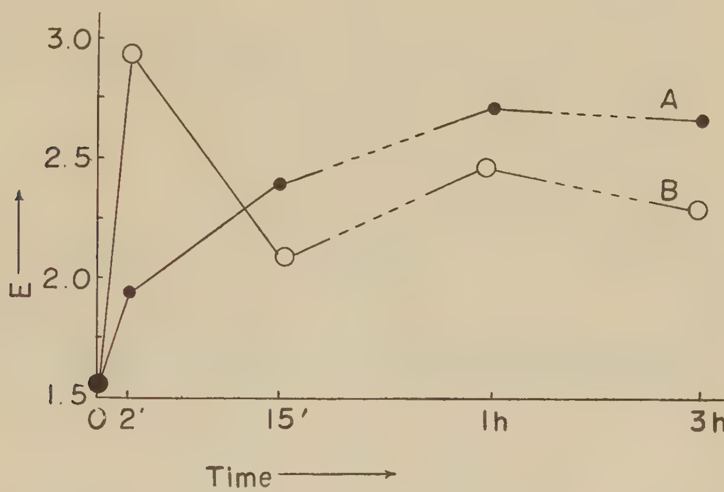


Fig. 9. Changes in color reaction given by activation and fertilization of the eggs of rainbow trout.

Unfertilized eggs, 2 min., 15 min., 1 hr. and 3 hrs. after activation and fertilization, were frozen with alcohol-dry ice mixture and dried in high vacuum. These samples were extracted with 10 times volume of water (regulated pH 7.0) under low temperature (below 2°C), and centrifuged at 15000 r. p. m. for 20 min. using refrigerated centrifuge. The reaction mixture consisted of 3 ml of supernates (sample), 2 ml of 1% Na_2CO_3 and 0.15 ml of 1% $CuSO_4$. The color reactions given by reaction mixture were measured by spectrophotometer using a filter of S_{57} (560mμ). Ordinates shows the optical density (E=−logT), and abscissa shows the time after activation and fertilization. Curve A indicates the changes in optical density after fertilization and curve B the changes in optical density after activation.

before, the reversible denaturation (renaturation) of egg protein was demonstrated by electrophoretic analysis because the opposite charged component appeared at the time of activation and then disappeared soon after. Although the reversible

denaturation of egg protein was not confirmed in this experiment, it may be concluded from the above data that the denaturation of egg protein does occur at the time of activation.

4) *Protease activities of the eggs in different stages after fertilization and activation*

According to Runnström ('49), fertilization reaction would involve the activation of several enzyme systems. In sea urchin eggs, G. Lundblad ('49) has demonstrated that such an activation occurs in vivo as far as proteolytic enzyme is concerned. In the first part of this section, the proteolytic activity of eggs in different fertilization stages has been measured. In the second part, is given a description of the activities of enzymes which split levetin and lipovitellin prepared from the egg. The third part of this section is concerned with some experiments on the confirmation of antitrypsin. The eggs of the pond smelt, crucian carp, rainbow trout and dog salmon are used as materials. As the principal aim of this work lies in a comparison of the activity of the same enzyme in different developmental stages, only relative values of activity have been recorded.

a) Proteolytic activity

The preparation of the egg and determination of enzyme activity were made in the same way as that described as having been employed in the first section of this chapter. In Fig. 10 A is shown the relative activity of gelatin-splitting enzyme in different stages of the fertilization of the pond smelt eggs, and in Fig. 10 B the activity of egg-albumin-splitting enzyme in different stages of the activation of the crucian

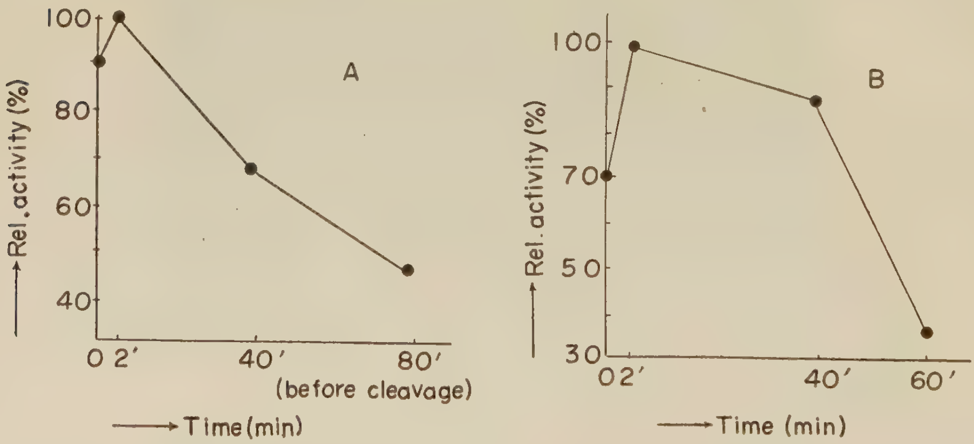


Fig. 10. Relative activity of proteolytic enzyme in different stages of development.
A : Gelatin-splitting activity of fertilized pond smelt eggs,
B : Egg-albumin-splitting activity of activated crucian carp eggs.

carp eggs. From these results, it is clear that there occurs a strong increase in activity of enzyme in a few minutes after fertilization or activation followed soon thereafter by a decrease in activity.

b) Levetin-, lipoprotein- and vitellin-splitting activities

It is physiologically significant to use the substrate prepared from the egg itself for the determination of enzyme activity of the egg. As stated above, levetin and lipoprotein were prepared respectively from ether extracted yolk. Vitellin fraction was prepared from lipovitellin mainly by Levene and Alsberg's method (cf. Akabori '43). Figs. 11, 12 and 13 show the changes in activities of these substrate-splitting enzymes along with the stages of the activated dog salmon eggs. Figs. 14, 15 and 16 also show the changes of activities in these enzymes with the advance of the fertilization process.

Levetin and vitellin splitting enzymes have increased in activity in a few minutes after activation or fertilization and thereafter they decreased in activity. Although the mode of action of enzymes is unknown, the change in activity of these enzymes appears to be similar to that of egg-albumin and gelatin splitting enzymes. On the other hand, lipoprotein-splitting enzyme has decreased in activity with the lapse of time after activation. Experiment made in the same way, using rainbow trout eggs, gave the same results as in the dog salmon egg.

c) Identification of antitrypsin-like substance

In the previous section, it was shown that proteolytic enzyme is present in the extracted from the eggs of pond smelt, crucian carp, dog salmon and rainbow trout.

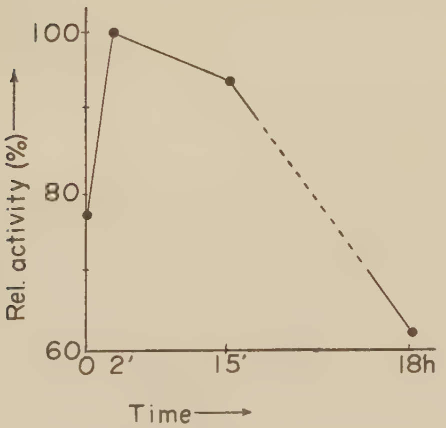


Fig. 11. Relative activity of levetin-splitting enzyme in activated dog salmon eggs.

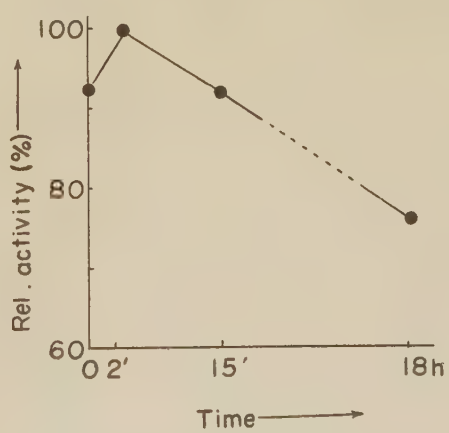


Fig. 12. Relative activity of vitellin-splitting enzyme in activated dog salmon eggs.

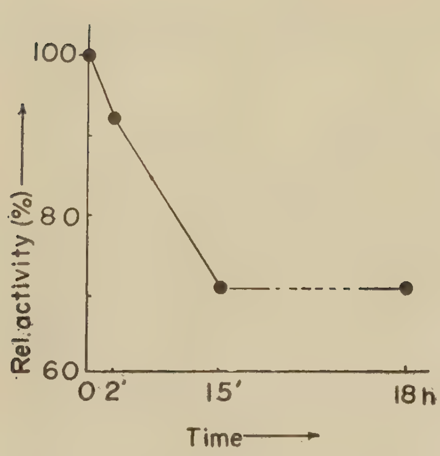


Fig. 13. Relative activity of lipoprotein-splitting enzyme in activated dog salmon eggs.

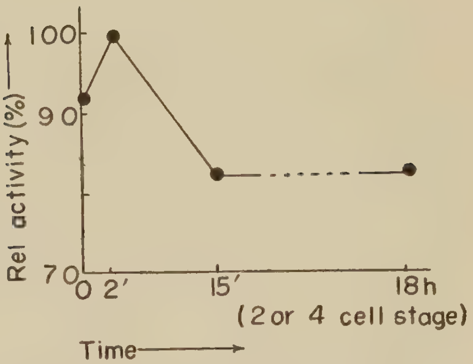


Fig. 15. Relative activity of vitellin-splitting enzyme in fertilized dog salmon eggs.

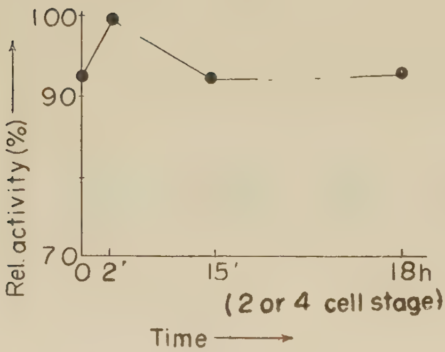


Fig. 14. Relative activity of levetin-splitting enzyme in fertilized dog salmon eggs.

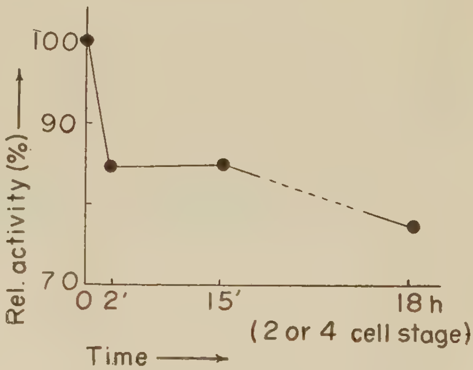


Fig. 16. Relative activity of lipoprotein-splitting enzyme in fertilized dog salmon eggs.

A few minutes after fertilization there appears a strong increase in activity of the enzymes thereafter followed by a decrease in enzyme activity. This blocking of activity of proteolytic enzymes in fertilization processes may be due to the presence of some enzyme inhibitor. Although the decrease in enzyme activity of the eggs at the time of activation can not be explained by the assumption of the presence of an enzyme inhibitor, the results presented in this section will become a first step of approach to this question. The trypsin inhibitor-like substance was prepared principally by Fredericq and Deutsch's method ('49) and by other methods such as those of Balls & Swenson ('34) and Lineweaver & Murray ('47). Among these methods,

Fredericq and Deutsch's yielded a fairly good result. Inhibitory potency of the enzymes was determined by comparing the activity of pure trypsin (Merk) with that of trypsin with inhibitor-like substance. Generally, the tryptic activity was measured by Anson's method ('38); however, KOH-alcohol titration or formol titration gave also identical results. As shown in Table 26, the results indicate that the trypsin inhibitor-like substance is present in the levetin fraction of ether extracted yolk. This

Table 26. Antitrypsin-like substance in dog salmon eggs.

Source prepared	Relative activity (%)
Control*	100
from levetin fraction	50
from ether extracted yolk of unfertilized eggs	120

* Control is represented by the casein-splitting activity by trypsin (Merk). Reaction mixture consisted of 1 ml of 0.1% casein (Hammarsten), 1 ml of buffer (ammonium buffer, pH 8.5), 0.5ml of 0.1% trypsin solution, and 1 ml of antitrypsin-like solution, and was incubated at 30°C for 2 hrs. To a blank test, the same volume of distilled water instead of antitrypsin-like solution was added. Trypsin solution was employed after dialysis for 12 hrs. at low temperature (below 5°C).

inhibitor was not detected in the inner swelling layer of egg membrane. The trypsin inhibitor was also not detected in ether extracted yolk itself. This may be due to disturbance by some substance in the course of preparation. Further, the antitrypsin-like substance showed a clear Fehling's reaction. Therefore, it seems reasonable to regard the inhibitor as a kind of ovomucoid which is also present in the hen egg (Fredericq & Deutsch '49, Lineweave & Murray '47). The antitrypsin-like substance probably plays a part in the blocking of the proteolytic activity in early developmental stages.

IV Role of sperm in fertilization

Since the time of Loeb who established the lysin theory, many authors have attempted to find a substance of enzymatic nature in the sperm suspension; they have attempted to extract from the suspension some substance effecting the unfertilized egg. Tyler ('39, '40) extracted from the sperm suspension a substance which dissolves the membrane of the limpet egg and called it a "lytic agent." Also an antisperm-agglutinin was extracted from the sperm suspension. In addition to Tyler's, there can be found many reports of physicochemical studies on the sperm. But such work was mainly done on marine invertebrates. Herein are reported some experiments which have been made on the sperm suspensions of dog salmon and rainbow trout. Firstly, the writer investigated the presence of an egg surface-liquefying substance

in the sperm suspension and then investigated the influence of this enzyme-like substance on the fertilization of the egg. Secondarily, some experiments concerning the possible relation between the sperm and the components of the egg protein (for example, levetin and lipovitellin) have been undertaken from the enzymatic standpoint.

1) *The egg surface-liquefying substance occurring in the sperm suspension of dog salmon*

Lundblad and Monroy, following Madinaveita's ('41) method employed for the preparation of mucinase from *Crotalus*, extracted the sperm of *Arbacia* and *Paracentrotus*, but this preparation had no effect on the viscosity of a solution of jelly coat substance. G. Lundblad showed further that the Madinaveita preparation contains a gelatin-liquefying enzyme (unpublished work, quoted by Runnström '49). The details of the present method are as follows. Of about thirty eggs, each one was cut into two parts respectively; they were divided into two groups, viz., control sample and experimental material. The error caused by lot can be avoided by this procedure. The control sample was washed with M/7.5 NaCl to remove yolk, and the membranes were dried at 110°C. The water content of the egg membrane was calculated from the weight of the wet and the dried egg membranes. In the experiment sample, yolk substance was removed in the same way as above. Next, the membranes were immersed in semen for 4 hours at 10°C, washed with M/7.5 NaCl to remove semen and then their water content was measured. If the surface-liquefying substance exists in the sperm suspension, the water content of the experiment sample must become smaller than that of the control one because the outer and inner layers of the chorion are dissolved out by immersion in semen. Therefore, the activity of the surface-liquefying substance in semen can be estimated from the difference between water content of both control and experiment.

a) Identification of surface-liquefying substance

The egg membrane samples were prepared by the above described method and were immersed in distilled water, in Ringer's solution and in semen respectively. As shown in Table 27, the surface-liquefying substance was not found in either the distilled water nor in the Ringer's solution samples, but it could be found in semen. Nextly, in order to determine the localization of this substance in semen, the following experiments were performed. The eggs were cut into three parts and three egg

Table 27. The surface-liquefying activity of sperm suspension.

Solution immersed	Water content of control (%)	Water content of experiment (%)	(Control)-(Experiment) (%)
in distilled water	64.4	64.7	− 0.3
in isotonic Ringer	63.5	63.4	+ 0.1
in semen	62.8	58.8	+ 4
	61.4	56.4	+ 5

membrane samples were prepared by the above described method. On the other hand, the semen was separated into seminal fluid and sediment by centrifugation. The first sample of egg membrane was used as the control. The second sample of the egg membrane was immersed in seminal fluid, and the third sample in sediment of semen. The results shown in Table 28 indicate that the surface-liquefying substance is present less in the sediment than in the seminal fluid. As has already been described in the chemical nature of the inner swelling layer of the dog salmon egg, the inner swelling layer and the hyaline layer of the egg membranes were removed by

Table 28. The localization of egg surface-liquefying activity in sperm suspension.

	Water content (%)
Control	67.7
Supernatant	63.3
Sediment	65.0
(Control) - (Supernatant)	+ 4.4
(Control) - (Sediment)	+ 2.7

treatment with N/50 HCl and the remaining chorions were washed with M/7.5 NaCl to remove HCl. These chorions were not affected by the surface-liquefying substance of sperm suspension. Thus it is certain that the surface-liquefying substance contained in sperm suspension acts upon the hyaline layer and inner swelling layer of the egg membranes.

Nextly, in order to investigate the influence of temperature on the surface-liquefying activity of sperm suspension, the semen was subjected to various degree of temperature and its activity upon membrane samples was examined. As shown in Table 29, the optimum temperature for the activity is approximately 10°C. The

Table 29. Influence of temperature on the surface-liquefying activity of sperm suspension.

Temperature	Water content of control (%)	Water content of experiment (%)	(Control)-(Experiment) (%)
0°C	65.4	64.3	+ 1.1
10°C	63.8	60.9	+ 2.9
20°C	64.3	63.6	+ 0.7
30°C	64.5	63.7	+ 0.8
100°C	65.1	65.1	0

surface-liquefying activity was markedly decreased in the semen heated at 30°C for 30 minutes and was lost entirely in the semen subjected to 100°C and 10 minutes treatment. The above findings show that the surface-liquefying substance has an enzymatic nature.

Further, the effects of various salt solutions upon the liquefying activity of sperm suspension were investigated, resulting the finding that NaCl, KCl and $CaCl_2$

have no effects on this enzyme (Table 30). On the contrary, when the sperm suspension was diluted with isotonic $MgCl_2$ and distilled water, the liquefying activity of sperm suspension was markedly decreased.

Table 30. Influence of salt solutions on the surface-liquefying activity of sperm suspension.*

Salt solution treated	Water content of control (%)	Water content of experiment (%)	(Control)-(Experiment) (%)
non-treated (control)	65.4	64.1	+ 1.3
dilute with distilled water	64.3	64.0	+ 0.3
NaCl	65.0	63.4	+ 1.6
KCl	66.5	64.0	+ 2.5
$CaCl_2$	65.1	63.9	+ 1.2
$MgCl_2$	64.8	64.7	+ 0.1

* A half volume of isotonic salt solution was mixed with the sperm suspension and this suspension was employed as the lytic solution.

b) Relation between the surface-liquefying substance and the hardening of the egg membrane

As has already been mentioned, the dog salmon eggs completely lose their fertilizability after a 2 minute treatment with water. Simultaneously, the hardening of the egg membrane occurs, owing mainly to the colloidal changes of the inner swelling layer of the egg membrane. The unfertilized eggs were immersed in water for various duration of time ranging from 30 seconds to 2 minutes. Egg membrane samples were prepared from these activated eggs by the method above described. These egg membrane samples were immersed in the sperm suspension and the liquefying-activity of the sperm suspension upon the egg membrane was investigated (Table 31). The liquefying substance present in sperm suspension did not act on the egg membrane treated with water for over 60 seconds. Thus the loss of fertilizability in eggs in

Table 31. The surface-liquefying activity of sperm suspension on water absorbed egg membranes.

Time absorbed water	Water content of control (%)	Water content of experiment (%)	(Control)-(Experiment) (%)
30 sec.	65.4	62.9	+ 2.4
40 sec.	65.5	64.4	+ 1.1
50 sec.	64.4	64.2	+ 0.2
60 sec.	64.6	64.5	+ 0.1
2 min.	63.5	63.3	+ 0.2

contact with water is correlated with the increase of resistant power of egg membrane to the liquefying-activity of sperm suspension. But the liquefying substance could have an influence upon the egg membrane weakly hydrolyzed with 10% HCl. In this case, the liquefying substance acts mainly on the middle layer of the egg membrane

because the inner and outer layers of the chorion have been dissolved out in the process of hydrolyzation. From the above, it may be concluded that liquefying-activity of sperm suspension decreases with the hardening of the egg membrane and this must be due to the changes in the nature of the egg membrane.

2) *Relation between the sperm and components of egg protein*

Maggio & Monroy ('55) demonstrated that phospholipids are released by the incubation of *Arbacia lixula* sperm with lipoprotein. This fact has been assumed to support the view that the cortical reaction to fertilization involves the splitting of a cortical lipoprotein complex. It is of interest to investigate the interaction between the sperm and the components of egg protein. Through the study of such a model test, some light may be thrown on the chain reaction leading to the activation of the egg. The present paragraphs deal with the proteolytic activity of sperm suspensisn upon components of egg protein and the behaviour of the spermatozoa suspended in these components. Detailed studies on proteolytic enzyme extracted from sea urchin spermatozoa have been made in recent years (Lundblad '54). In such enzymatic studies, gelatin, casein, haemoglobin, etc. were employed as the substrate. But in the present study, levetin and lipovitellin, to be used as substrate, were prepared from unfertilized egg yolk by the same method as described in the fractionation of egg protein of the rainbow trout. Then the substrate-splitting activities of sperm extracts were measured. The detailed procedures are described in the foot note to Table 32. As indicated in Table 32, the levetin-splitting enzyme of sperm suspension showed stronger activity than the lipovitellin-splitting enzyme does. On the contrary, the trypsin acted more strongly on lipovitellin fraction than on levetin fraction (Table 33).

Table 32. Levetin- and lipovitellin-splitting activity of sperm suspension.

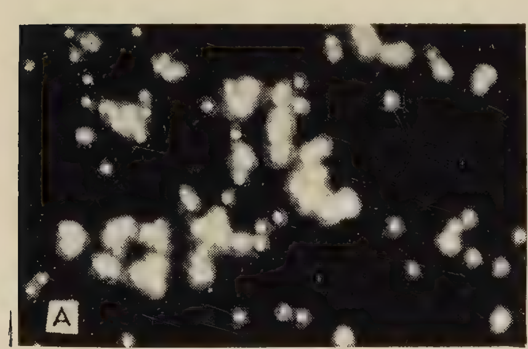
	Exptl. No.	mg of released amino N from 1 ml of substrate/hour*
Levetin-splitting activity of sperm suspension	1	0.34
	2	0.69
	3	0.34
	4	0.27
Lipovitellin-splitting activity of sperm suspension	1	0.19
	2	0.19
	3	0.27

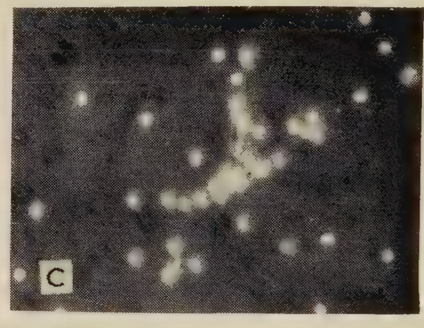
* Precipitated levetin (or lipovitellin) was dissolved in M/7.5 NaCl, in which protein concentration was about 1 per cent. Reaction mixture consisted of 1 ml of substrate, 1 ml of buffer solution (veronal buffer, pH 6.9) and 0.5 ml of sperm extract, and was incubated at 30°C for 2 hrs. Released amino nitrogen was measured by micro-titration of KOH-alcohol solution. Fresh dry semen was diluted with three volumes of distilled water (adjusted to pH 7.0) and centrifuged. The supernates were used as enzyme solution of the sperm suspension.

Table 33. Levetin- and lipovitellin-splitting activity by trypsin.

	Exptl. No.	mg of released amino N from 1 ml of substrate/hour*
Levetin-splitting activity	1	0.81
	2	0.98
	3	0.44
Lipovitellin-splitting activity	1	1.49
	2	2.46
	3	2.24
	4	1.39

* Substrates were prepared by the same method as described in Table 32. Reaction mixture consisted of 1 ml of substrate, 1 ml of buffer solution (veronal buffer, pH 8.2) and 0.5 ml of 0.1% trypsin solution, and was incubated at 30°C for 2 hrs. Released amino nitrogen was measured by micro-titration of KOH-alcohol solution.





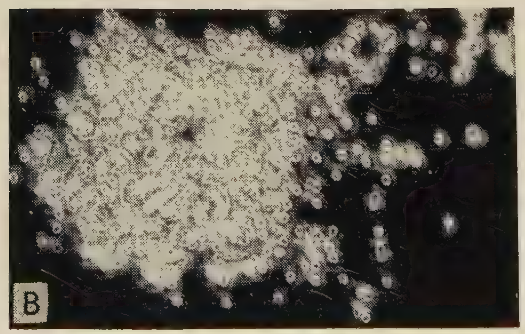


Fig. 17. Sperm-agglutination in egg protein components.
A : Sperm-agglutination in levetin solution, (Ca. × 120)
B : Sperm-agglutination in lipovitellin solution, (Ca. × 120)
C : Sperm-agglutination in M/7.5 NaCl. (Ca. × 120)

Making another biological approach, the writer made microscopic observation of sperm agglutination in these components of egg protein. In this experiment, the sperm of rainbow trout was used as material. Levetin and lipovitellin fractions from egg yolk of the rainbow trout were dissolved in M/7.5 NaCl and two or three drops of semen were added to these protein solutions. The sperm-agglutinating reactions were observed under a phase contrast microscope. As control, the sperm-agglutination

reaction in M/7.5 NaCl was observed in the same manner. Strong sperm-agglutination was found to occur in lipovitellin solution. But such a mass formation was not found in levetin solution in which, however, the same degree of sperm agglutination as in M/7.5 NaCl was discernible (Fig. 17).

The dog salmon sperm also gave the same results as above.

Although no other data concerning the response of the spermatozoa to the egg protein components have been gathered, it may be expected that there exists a certain correlation between proteolytic activity of sperm suspension and sperm-agglutinating activity of the egg portein component.

GENERAL DISCUSSION

The envelope of the dog salmon egg consists of three layers, an outer hyaline layer, a middle layer and an inner swelling layer. The middle layer is composed of kerato-elastin, while the inner swelling layer as well as the outer hyaline layer is composed of glycoprotein. Many works have already been undertaken on the chemical nature of the egg membrane in various kinds of animals. The surface gelatinous substance of the amphibian eggs consists almost entirely of mucoprotein and mucin has been isolated from that gelatinous substance (vide Needham '31). The jelly coat of sea urchin eggs is also rich in mucin; in the coat the presence of carbohydrate component has been demonstrated (Immers 52, Nakano '54). In fish eggs, little attention has hitherto been paid to the changes taking place in the egg membrane at the time of fertilization. The fact that soft egg membrane gradually becomes hard at fertilization has merely been reported. Kusa ('49a, '49b) has suggested in the case of the salmon egg that the egg membrane hardens as the result of water absorption by the egg, and that ionic calcium which originates from the body fluid plays an important role in the hardening of the egg membrane. It is known that colloid substance, such as gum arabic, gum tragacanth, albumin and gelatin, can induce hardening of the egg membrane (Kusa '49a, Nakano '56). On the other hand, in the sea urchin egg, Monroy & Runnström ('48) have suggested that disulfide bonds contribute to the hardening of the fertilization membrane. As shown in the present paper, the inner swelling layer of the dog salmon eggs untreated in water readily swells up in acid solution and finally disperses in the solution, whereas the layer in contact with water can be removed from the chorion as a white film layer. The egg membrane which absorbed water increases in Ca-content and changes in colloid character. These changes in the inner layer may exert an influence on the hardening of the egg membrane. Therefore, it may be concluded that the hardening of egg membrane is mainly attribute to the colloidal change of the inner layer induced by the absorption of calcium ions.

The lytic substance which behaves like sperm lysin from the sea urchin sperm was isolated from mature mackerel testis (vide Runnström '49). Runnström ('32) stated that an inhibitor of proteolytic enzyme was present in the vitellin membrane and

cortical layer of the sea urchin egg. The inhibitory action is assumed to be removed, according to him, by a substance present in the sperm. The dog salmon sperm also contains some lytic substance which dissolves the inner swelling and the outer hyaline layers, but not the hardened inner layer. But no trypsin inhibitor was demonstrated in the outer and inner layers of the egg membrane. Therefore, the inactivation of a lytic enzyme on the hardened egg membrane must be due to the changes in the properties of the egg membrane rather than to any presence of an inhibitor as has already been shown in the sea urchin egg.

The cluster formation of the dog salmon sperm is induced by addition of the surface gelatinous substance of the egg which is dissolved out in acid solution. From these facts, a fertilizin-like substance is expected to be contained in surface gelatinous layer, because the gelatinous substance exerts the same effect on the spermatozoa as a fertilizin of the sea urchin egg does.

Hartman *et al.* ('47) made the interesting observation that an inhibitor of fertilizin is present in *Salmo* egg. This agrees with Lillie's ('19) observations concerning the presence of an antifertilizin in the sea urchin egg. From the above, it seems very interesting that the fertilizin and antifertilizin-like substances are also detected in the fish egg and sperm.

Runnström ('49) held the hypothesis that incitment of development may be due to activation of several enzyme systems. On this point, many data have already been gathered in sea urchin eggs. Ishida ('49) advanced an opinion holding that the activation of the egg is accompanied with the activation of some kind of esterase and that the negative wave thus induced may sweep over the plasma surface of the egg in an instant. Yamamoto ('49) suggests that the esterase-like substance present in the minute cortical granules may participate in the activating reaction. The present study shows that crucian carp eggs are activated by an esterase-activator and that the activation of the egg is delayed by an esterase-inhibitor. Further, some esterase-like substance is detected in the egg. Although it has not yet been clarified completely whether the esterase-like substance certainly participates in the activating processes or not, it is highly probable that it does, taking into consideration Yamamoto's observation and Ishida's results.

In the mackerel egg, Harvey & Shapiro ('34) and Danielli & Harvey ('35) have investigated the denaturation of the egg protein by measurement of interfacial tension between the aqueous contents and egg oil. Heilbrunn ('36) ascertained that sea urchin eggs treated with dilute solution of ammonium salts increase in free fat or lipid, and asserted that this may be caused by the alkalization of the protoplasm. As reported in the present study, when the unfertilized dog salmon eggs are kept at low temperature (below 4°C), the large oil drops increase in diameter, while the oil granules decrease in number. Simultaneously, the total amount of oil increases. Although knowledge concerning the chemical mechanism of the increase in amount of

oil of the egg is still very meagre, the following assumption may be possible: that the protein-lipid binding of the lipoprotein present in the egg is probably split by denaturation of egg protein and results in the release of free lipid. Further, the total amount of oil of the dog salmon egg is increased by treatment with ammonium chloride solution. In this case, the protein-lipid binding of lipoprotein may also be affected by treatment with that solution. Then, in parallel with these changes in egg oil, the fertilization capacity of the egg decrease suddenly.

In the living organism certain enzyme system may exist naturally in the inactive or zymogen state and become active as a result of a change of pH or of the action of some other enzyme.

It has recently been reported by Lundblad & Hultin('54) that two proteolytic enzymes are contained in the extraction of sea urchin eggs and can be liberated by ribonuclease. It seems possible that the enzymes of the unfertilized egg are present in inactive state and that the inactive enzymes are activated indirectly by fertilization or activation. This idea is supported by the experimental fact that the inhibitory effect of mercury bichloride has been counteracted by hydrocyanide. Further support is afforded by the facts that the various kinds of protease were contained in fish eggs and that at activation they were affected by protease-activator or -inhibitor as in the case of esterase of the crucian carp egg. On the other hand, few minutes after fertilization there happens a strong increase in protease activity which was followed by a decrease in activity. There are no data at present available for the explanation of this phenomenon. But from the facts that Ca ions and fatty acid act on the enzyme of the egg as protease inhibitors and that antitrypsin-like substance can be demonstrated in the eggs of dog salmon, it may be supposed that these inhibitors take part in the blocking of the enzyme. The following items of evidence seems also to support this supposition, viz., Ca ions are necessary for the fertilization process and also fatty acid probably originates from the decomposed substance of esterase as has been proven in the crucian carp egg.

Changes in protein pattern of the egg which occur as the result of fertilization were demonstrated by Mirsky('36). He found that the protein fraction of the unfertilized sea urchin egg becomes insoluble during first 10 minutes after fertilization. In this regard, Loeb ('13) working on the sea urchin egg first proposed a theory that the first period of fertilization or parthenogenesis stimulates the elevation of the membrane by cytolysis of the cortex of the egg. Later, Rapkine('30) demonstrated the increase of -SH groups at the time of fertilization in the sea urchin eggs. However, according to Mirsky('36), these changes in protein pattern are not accompanied by a liberation of the -SH groups, being more like the phenomena related to the reversible denaturation of myosin during muscular contraction. Recently, the electrophoretic analysis of water extract of sea urchin eggs has been made by Monroy ('50). He has reported that a new component appears after fertilization but disappears soon after.

In the present experiment, the electrophoretic analysis of activated eggs shows the occurrence of an opposite charged component which appears after activation and disappears soon after. On the contrary, in fertilized egg, this component can not be discovered. It is difficult to say what kind of reaction takes part in changes of protein level which happen soon after activation. But it seems probable that these changes may be concerned with a weak degree of denaturation of protein. The occurrence of protein denaturation at the time of activation was also proven by biuret reaction. As shown by the electrophoretic analysis, the degree of denaturation is stronger in activated eggs than in fertilized eggs. But in activated eggs the protein denaturation may be overcome to some extent within a short time, because the opposite charged component disappears soon after activation. Together with these changes, the egg loses its fertilizability. From the above evidence, it can be said that there exists a correlation between the appearance of the opposite charged component and the lose of fertilizability. Further, from the experimental finding that the opposite charged component can be detected in the lipovitellin fraction, it seems to be certain that the cortical reaction of activated eggs involves the denaturation of the lipovitellin fraction. Thus the denaturation of egg protein might be regarded as the first steps in the chemical changes which occur in the process of activation. In other words, egg activation is accompanied by a reorganization of the pre-existing protein pattern.

The agglutination of spermatozoa is different in different protein fractions of egg protein and the splitting activity of the spermatozoa is more effective on levetin than on lipovitellin. These facts show that the cortical layer of the egg is probably more resistant to entrance of the spermatozoa than the inner layer of the egg is. On the contrary, the splitting activity of trypsin acts more strongly on lipovitellin than on levetin. The different sensitivity of trypsin shown in the present experiment may be due to some trypsin inhibitor which is present in the levetin fraction of egg protein. In this field of studies, the sensitivity of extracts of unfertilized and newly fertilized eggs of *Arbacia lixula* to trypsin and to urea has already been reported by Giardina & Monroy('55) and Ceas, Impellizzeri & Monroy('55).

SUMMARY

The physico-chemical characters of fish eggs at the time of activation or fertilization have been investigated, and the following results were obtained.

1) The envelope of the dog salmon egg consists of three layers, an outer hyaline layer, a middle layer and an inner swelling layer. The inner swelling layer was found to be mainly composed of glycoprotein like a kind of mucoid. The inner swelling layer which has fully absorbed water was richer in Ca-content than that which was not in contact with water ; there exists a clear parallel relationship between this increase of Ca and the change in colloidal character of the egg membrane, which may indirectly influence the hardening of egg membrane at the time of fertilization or activation.

2) The hyaline layer, the inner layer and semen showed respectively a different precipitation reaction to salt, acid and alkaline solutions. It seems probable that these substances play a different roles respectively at the time of conjugation between eggs and spermatozoa. The egg fluid appears also to play a role in the conjugation of eggs and sperm.

3) The presence of the Ca ions was necessary for the fertilization processes. The Ca of semen appeared not to take part in the fertilization, but the Ca of the chorion and perivitellin space was needful for the fertilization of eggs.

4) When ripe unfertilized eggs of crucian carp were treated with Ringer-esterase activator and returned to isotonic Ringer's solution, the egg was activated. On the contrary, the activation of the egg was delayed by treatment with Ringer-esterase inhibitor. Moreover, the presence of an esterase-like enzyme in the egg itself was proven.

5) In dog salmon eggs, minute oil granules decreased in number along with increase in size of larger oil drops; the amount of oil increased in total. A similar increase of oil was observed in eggs immersed in NH_4Cl-, 0.5% saponin Ringer-, various pH (pH 5, 8), alcohol Ringer- or ether Ringer-isotonic solutions as well as in eggs treated with heat (at 20°C). On the contrary, the amount of oil was decreased by treatment with 0.5% chloreton-Ringer. Isotonic salt solutions (NaCl, KCl, $CaCl_2$ and $MgCl_2$) exerted no effects on the oil volume of eggs.

6) Protease activity was found in extracts from the eggs of pond smelt and crucian carp. Protease-inhibitor and -activator influenced the fertilization or activation of the eggs. Among the various enzyme inhibitors, mercury bichloride exerted a harmful effect on the fertilization of the pond smelt eggs. But this effect could be removed by treatment with hydrocyanide. The activating effect of lead-acetate on the egg was also removed in part by hydrocyanide.

Glycyl-glycine dipeptidase activity of the crucian carp egg was inhibited by fatty acid and Ca ions which were essential for fertilization processes.

7) Electrophoretic analysis was made of the activated and fertilized eggs in the pond smelt, dog salmon and rainbow trout. In activated eggs, the opposite charged component appeared in the lipovitellin fraction of egg protein and disappeared soon after. On the contrary, in fertilized egg, this opposite charged component did not appear.

8) No lysozyme-like substance could be detected in this experiment. Also, this opposite charged component did not appear as a result of interaction between levetin-like fraction and water soluble protein fraction.

9) The electrophoretic diagrams of heated ether extracted yolk and heated lipovitellin fraction from unfertilized eggs closely resemble those of activated and fertilized eggs.

10) The coloration given by biuret reaction became more intensive with the

lapse of time after activation or fertilization.

11) Gelatin and egg-albumin-splitting enzymes of the eggs of pond smelt and crucian carp increased in activity in some minutes after activation or fertilization and then the activity decreased gradually. The levetin- and vitellin-splitting enzymes of the dog salmon eggs showed similar activity to that of gelatin- and egg-albumin-splitting enzymes. On the other hand, the antitrypsin-like substance was found in the levetin fraction of the dog salmon eggs.

12) The egg membrane-liquefying substance was detected in the sperm suspension of dog salmon. This substance did not act on the middle layer of egg membrane but on the inner swelling layer. It existed mainly in the seminal fluid. The optimum temperature for the activity was approximately 10°C, and the substance readily became inactive as a result of heating. NaCl, KCl and $CaCl_2$ have no effects on this liquefying activity. The lytic substance present in sperm suspension did not act on the egg membrane of activated eggs.

13) The presence of levetin- and lipovitellin-splitting enzymes was demonstrated in the sperm suspension of rainbow trout; the former showed stronger activity than the latter.

14) Sperm suspension of the rainbow trout was agglutinated strongly in lipovitellin-NaCl solution, but such a mass formation of the sperm suspension could not be detected in levetin-NaCl solution.

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